AGE-RELATED IMPAIRMENTS IN MEMORY AND IN CHOLINERGIC SIGNALING DUE TO REDUCED BLOOD GLUCOSE RESPONSES TO EPINEPHRINE

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

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ABSTRACT

Memory impairments accompany aging and can range in severity from mild deficits during healthy aging to debilitating conditions such as Alzheimer's disease. The causes of these impairments are generally viewed in terms of anatomical, chemical, and physiological changes within the brain. However, age-related changes in peripheral neuroendocrine systems can alter central neurobiological processes and may underlie some types of memory deficits. Previous work in rodents and humans indicates that increases in blood glucose in response to endogenous epinephrine release are important for regulating memory formation and durability. In old rats, glucose is less responsive to epinephrine and becomes severely depleted in the extracellular fluid of the hippocampus during a cognitively demanding task, contributing to poor memory. Glucose supplementation restores extracellular fluid levels of glucose and reverses the associated memory impairments.

The present work examines potential mechanisms by which reduced blood glucose responses to epinephrine may produce age-related memory impairments, with particular emphasis on the ability of exogenously administered epinephrine or glucose to attenuate deficits in memory processes. Several findings suggest fluctuations in glucose levels in the extracellular fluid may modulate memory processes by altering the release of the neurotransmitter acetylcholine, which may affect downstream processes important for the formation of durable memories. In particular, nicotinic acetylcholine receptor signaling mediates the calcium-dependent activation of the ERK/MAPK and CREB signaling cascades, which are widely implicated in activity-dependent neuronal plasticity. Age-related deficits in brain glucose availability may limit these cholinergic

signaling processes and produce memory impairments. This is the major hypothesis guiding these studies.

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ACKNOWLEDGEMENTS

I would especially like to thank my advisor Dr. Paul Gold for his guidance and support during my graduate studies. None of this work would have been possible without his inspiration, ideas, and track record of influential research that served as the basis for these experiments. I would also like to thank my unofficial “co-advisor” Dr. Donna Korol for her invaluable perspective and willingness to challenge various aspects of my research. I appreciate the participation of my committee members, Drs. Lee Beverly, Hee Jung Chung, and Roberto Galvez, as well as their input into the nicotinic experiments.

I would like to acknowledge those who made significant contributions to this work. I shadowed Dr. Qing Chang early in my graduate career, and he performed much of the behavioral and neurochemical analyses for the experiments in Chapter 2. Dr. Eric Mohler performed blood glucose measurements for the experiments in Chapter 2. Sisi Li and Duat Bui assisted in the nicotinic experiments in Chapter 5.

Finally, I am grateful to all of the Gold-Korol lab undergraduates, technicians, graduate students, and post-doctoral research associates who I have had the privilege of working with over the years. The collaborative atmosphere and professional environment in the lab truly enhanced my graduate experience.

This work was supported in part by a National Research Service Award (F30AG034803) from the National Institute on Aging.

TABLE OF CONTENTS

CHAPTER 1: BACKGROUND AND SIGNIFICANCE 1

CHAPTER 2: AGE-RELATED MEMORY IMPAIRMENTS DUE TO REDUCED BLOOD GLUCOSE RESPONSES TO EPINEPHRINE 27

CHAPTER 3: AGE-RELATED IMPAIRMENTS IN MEMORY AND IN CREB AND pCREB EXPRESSION IN HIPPOCAMPUS AND AMYGDALA FOLLOWING INHIBITORY AVOIDANCE TRAINING 50

CHAPTER 4: EPINEPHRINE AND GLUCOSE MODULATE TRAINING-RELATED CREB PHOSPHORYLATION IN OLD RATS: RELATIONSHIPS TO AGE-RELATED MEMORY IMPAIRMENTS 76

CHAPTER 5: GLUCOSE ATTENUATES IMPAIRMENTS IN MEMORY AND CREB ACTIVATION PRODUCED BY AN α4β2 BUT NOT AN α7 NICOTINIC RECEPTOR ANTAGONIST 106

CHAPTER 6: CONCLUSIONS 136

REFERENCES 142

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

1.1. AGE-RELATED FORGETTING

In humans, memory impairments accompany healthy aging as well as various age-related pathologies, which can range in severity from Mild Cognitive Impairment to debilitating conditions such as Alzheimer's disease. A variety of age-related memory impairments manifest as the rapid forgetting of recent information and events, with older memories remaining intact (Craik, 1994; MacDonald et al., 2006; Munro Cullum et al., 1990; Park et al., 1988; Wheeler, 2000). Age-related rapid forgetting may develop gradually throughout the lifespan and is task-dependent, with certain types of information more susceptible than others. For example, tests of free recall of word lists, verbal passages, and pictures are much more sensitive to aging effects than those involving only recognition of previously encountered items. Age-related forgetting is also particularly evident in tests of spatial navigation and working memory tasks. In most of these tasks, forgetting is evident even when learning is matched between young and old subjects, indicating that forgetting does not erroneously reflect age-related differences in attention or acquisition.

Similar findings of accelerated forgetting have been reported in rats and mice, with specific time courses that differ by age and task (Barnes, 1979; Barnes and McNaughton, 1985; Gold, 2005; Korol, 2002; Winocur, 1988). For example, a commonly used rodent model of age-related forgetting is inhibitory avoidance training. In this task, a rodent is placed into a small box illuminated by a bright light and is allowed to cross over freely into a dark chamber, where it receives a foot shock. At a later time, usually several hours or days later, the rodent is again placed into the light chamber and its

testing retention latency to cross over into the dark chamber is recorded. The testing retention latency is taken as a measure of memory for receiving the foot shock, with longer latencies indicating good memory and shorter latencies indicating forgetting. In young adult rats, memory for inhibitory avoidance training is stable for up to three weeks following a brief, mild foot shock. However, old rats exhibit slight deficits after one day, and are significantly impaired after seven days. These results cannot be explained by a lower sensitivity to the foot shock in old compared to young rats, as old rats have similar perceptual thresholds as young rats for the same foot shock and have comparable or even better memory than young rats when tested two hours after training. Thus, old rats exhibit rapid forgetting for inhibitory avoidance, with memory intact for hours after training, but with memory impairments emerging during the days after training (Gold et al., 1982). A major advantage of inhibitory avoidance training is that it tests memory after a single learning trial, thus eliminating confounds due to multiple trials. Another advantage is drugs that modulate memory formation can be administered post-training, eliminating the effects of these drugs on perception, motivation, or other factors that might alter learning instead of memory.

Another good model of age-related forgetting is the spontaneous alternation task—a test of spatial working memory. In this task, a rodent is allowed to freely traverse a three- or four-arm maze in a room containing a variety of spatial cues. A rodent with good spatial memory will tend to visit arms it has not recently visited. This tendency can be quantified and compared to chance performance on the task. For example, in the four-arm maze, visiting each of the four arms in overlapping sets of five attempts constitutes an alternation. Young adult rats trained on this task generally have

average alternation scores between 55% and 65%, which is significantly better than chance performance (44%). Though old rats visit similar numbers of arms as young rats, their alternation scores are at or near chance performance, indicating significant age-related forgetting (McNay and Gold, 2001). Increasing task difficulty by instituting a delay between arm entries impairs performance in young adult and middle-aged rodents, which is useful for modeling aging effects (e.g. Stone et al., 1997). In contrast, reducing task difficulty by utilizing a three-arm instead of a four-arm maze attenuates age-related impairments (McNay and Gold, 2001). Spontaneous alternation training has an advantage over similar tasks because it is non-appetitive and does not require associated rewards (food, water) or punishments (e.g. foot shock, immersion in water) to motivate performance.

The present studies utilize both inhibitory avoidance and spontaneous alternation training to model age-related forgetting. Besides these tasks, rodents exhibit age-related forgetting in a variety of other tasks, including the water maze (Burke et al., 2008; Gage et al., 1984; Mabry et al., 1996; Rapp et al., 1987), reward reduction (Salinas and Gold, 2005), social transmission of food preference (Countryman and Gold, 2007), visual discriminated avoidance (Gold et al., 1982), Barnes circular maze (Barnes and McNaughton, 1985), spatial reversal (Zornetzer et al., 1982), odor-reward association (Roman et al., 1996), and eye-blink classical conditioning (e.g. Solomon et al., 1995; Woodruff-Pak et al., 2007). Thus, rapid forgetting is a key characteristic of age-related memory impairments.

Previous work in rodents suggests rapid forgetting may reflect deficits in neurobiological mechanisms of memory formation initiated during or soon after training.

For example, old rodents have training-related alterations in neurotransmitter release, calcium signaling, and gene expression within the brain, all of which may be interrelated and contribute to memory impairments (Burke and Barnes, 2006; Dickstein et al., 2007; Kelly et al., 2006; Mora et al., 2007; Toescu and Verkhratsky, 2007; Yankner et al., 2008). These age-related alterations do not all necessarily reflect intrinsic deficits in brain function, but may originate from changes in peripheral processes. A variety of evidence suggests that changes in peripheral hormones and neuroendocrine systems during aging can alter central neurobiological processes, producing deficits in memory and synaptic plasticity (Conrad and Bimonte-Nelson, 2010; Foy, 2011; Frick, 2009; Janowsky, 2006; Korol and Gold, 2007; Lupien et al., 2009).

1.2. EPINEPHRINE, GLUCOSE, AND RAPID FORGETTING

Of particular relevance to the work presented here, numerous studies suggest that deficits in blood glucose responses to epinephrine during and after training may contribute to age-related impairments in memory processes (Gold, 2005; Korol, 2002; Korol and Gold, 1998; Messier, 2004). In non-aged populations, the pathway through which glucose modulates memory processes in response to endogenous epinephrine release is one of the most extensively studied neuroendocrine systems involved in learning and memory (Gold, 1995, 2008; Gold and Korol, 2010; Korol and Gold, 2007; Messier, 2004). Epinephrine, which is released from the adrenal medulla in response to emotional or stressful events, enhances memory for a variety of tasks in both rodents and humans. The enhancement follows an inverted-U dose-response curve in which moderate doses of epinephrine enhance memory and high doses impair memory. Likewise, epinephrine enhances the duration of long-term potentiation (LTP), which is

widely considered to be an analog of memory, from minutes to at least several days, following a similar inverted-U dose-response curve (Korol and Gold, 2008). Since epinephrine does not readily cross the blood brain barrier, the hormone most likely enhances memory by engaging peripheral mechanisms that modify brain functions. Two major mechanisms have been examined for how epinephrine enhances central memory processes. One mechanism proposes that epinephrine activates vagal afferents projecting to the nucleus of the solitary tract (NTS). Adrenergic activation of NTS neurons, in turn, potentiates release of norepinephrine in the amygdala and enhances performance in emotionally arousing tasks (McGaugh, 2004; McGaugh and Roozendaal, 2002; McGaugh et al., 2002). Another mechanism, which is the major focus of the present work, proposes that glucose is a major intermediary of the effects of epinephrine on memory. Glucose is released from liver glycogen stores in response to circulating epinephrine. Like epinephrine, peripheral injections of glucose enhance memory in rodents and humans, also following an inverted-U dose-response curve. Peripheral adrenergic receptor antagonists block the memory-enhancing effects of epinephrine injections (Sternberg et al., 1985, 1986) but not of glucose (Gold et al., 1986), supporting the view that glucose bypasses this step in the memory enhancement pathway. Moreover, direct infusions of glucose into the lateral ventricles or into specific brain areas, including the medial septum, hippocampus, and amygdala, enhance memory, which is consistent with the idea that glucose acts directly on brain mechanisms of memory (Gold, 2005). Thus, the efficacy of direct brain glucose injections in enhancing memory supports the view that blood glucose modulates memory processes by regulating brain glucose levels.

Prior work indicates there are age-related declines in blood glucose responses to epinephrine, which may contribute to memory impairments by limiting the supply of glucose to the brain during cognitively demanding times (cf. Gold, 2005). Studies by Mabry et al. (1995a,b,c) indicate that plasma epinephrine levels increase more in old compared to young rats in response to acute swim stress, chronic restraint stress, and foot shock. In contrast, plasma glucose levels increased in young but not old rats shortly after foot shock (Mabry et al., 1995c). Thus, endogenous epinephrine release in response to foot shock has little effect on blood glucose levels in old rats. Other studies have shown that regional fluctuations in brain glucose concentrations in the extracellular fluid are important in memory processing, and that these fluctuations have important consequences during aging. When rats perform a spontaneous alternation task, the levels of extracellular glucose in the hippocampus decrease significantly. Systemic injections of glucose block this decrease and enhance memory for the task (McNay et al., 2000). This suggests there is a limited supply of glucose to be taken up and utilized by hippocampal cells in support of memory processes. Aged rats exhibit significantly greater forgetting combined with a much larger and longer duration of reduction in extracellular glucose levels compared to young rats. The more rapid recovery of brain glucose levels in young rats is associated with a rise in blood glucose levels, which is absent in aged rats. Of particular interest, peripheral injections of glucose reverse the age-related depletion of hippocampal glucose and improve memory scores of aged rats to those of young rats (McNay and Gold, 2001). Together, these results indicate that age-related declines in blood glucose rises in response to training/epinephrine may cause large reductions in hippocampal glucose and associated memory impairments.

Age-related deficits in glucose availability within the brain may contribute to memory impairments through a variety of mechanisms. Glucose utilization by the pancreas provides one model for how glucose may function in the brain and how deficits in central glucose availability may produce memory impairments. Pancreatic islet cells have a large number of ATP-dependent potassium (K-ATP) channels that are sensitive to subtle changes in the ATP/ADP ratio. Increased glucose metabolism due to higher extracellular glucose increases the ATP/ADP ratio, facilitating membrane depolarization by closing of K-ATP channels. Membrane depolarization promotes activation of voltage-dependent calcium channels. The resulting influx of calcium into the cell is the main trigger for insulin release, which ultimately reduces blood glucose levels (MacDonald and Wheeler, 2003). Increases in intracellular calcium levels also lead to activation of a variety of calcium-mediated signaling pathways, altering gene expression and promoting islet cell proliferation and survival. The protein CREB regulated transcriptional coactivator 2 (CRTC2) is an important mediator of these changes in gene expression (Jansson et al., 2008; Screaton et al., 2004).

Similar to the pancreas, the brain contains a large number of K-ATP channels that are responsive to local variations in extracellular glucose levels. K-ATP channels in the brain likely modulate the release of neurotransmitters and promote changes in gene expression through calcium-dependent mechanisms. A number of studies indicate K-ATP channel blockers promote memory processes and may mediate the effects of glucose on memory enhancement (Ghelardinia et al., 1998; Rashidy-Pour, 2001; Stefani and Gold, 1998, 2001; Stefani et al., 1999). There are several different CRTCs expressed in the brain, and CRTC1 is important in the generation of hippocampal LTP

(Alberini, 2009; Kovacs et al., 2007; Wu et al., 2007). Together, these results suggest that age-related deficits in glucose supply to the brain may inhibit the ability of glucose to regulate neural excitability and modulate molecular memory processes through actions at K-ATP channels. Although this is an intriguing possibility, there are some inconsistencies regarding the role of K-ATP channels in glucose-mediated memory formation. Stefani and Gold (2001) found that the K-ATP channel opener lemakalim impaired, whereas the K-ATP blocker glibenclamide improved, spontaneous alternation performance, as predicted. However, both lemakalim and glibenclamide enhanced hippocampal acetylcholine output, indicating a dissociation between K-ATP channel modulators on memory and acetylcholine release. As will be discussed below, acetylcholine is thought to be a major mediator of glucose's effects on memory processes. This discrepancy suggests that modulation of K-ATP channels initiates mechanisms parallel to or downstream of acetycholine's effects on memory processes.

Besides working through K-ATP channels, glucose may modulate other neurochemical or cellular processes important for memory enhancement. Dash et al. (2006) implicated the mammalian target of rapamycin (mTOR) signaling cascade, showing that direct glucose administration into the dorsal hippocampus improved memory in the water maze task while activating downstream markers of mTOR activity. Further, glucose failed to reverse memory impairments induced by intrahippocampal rapamycin. However, a major problem with the Dash study is that rapamycin administration obliterated phosphorylation of several proteins involved in the mTOR signaling cascade, reducing their expression well below basal levels. Thus, rapamycin may have impaired memory by altering normal cellular functions rather than by simply

inhibiting training-initiated cell signaling processes. In addition, although glucose activated proteins in the mTOR pathway, it had no effect on activation of mTOR itself.

1.3. GLUCOSE MODULATION OF ACETYLCHOLINE

Perhaps the most widely studied mechanism for glucose's effects on memory processes, which is not necessarily mutually exclusive of those mechanisms already discussed, is through modulation of the neurotransmitter acetylcholine. Acetylcholine is widely implicated in learning and memory processes, activating a wide range of signaling pathways that are responsive to diverse stimuli. One of the most consistent findings in aging and age-related memory pathologies is alterations or reductions in cholinergic signaling processes (Bartus, 2000; Schliebs and Arendt, 2011; Terry and Buccafusco, 2003). These deficits are most evident in later stages of Alzheimer's disease, in which there is clear degeneration in cholinergic neurons in the basal forebrain. However, there is a general preservation in cholinergic cells during normal aging, Mild Cognitive Impairment, and early stages of Alzheimer's disease. In these cases, impairments in cholinergic function are evident by alterations in acetylcholine release, high-affinity choline uptake, nicotinic acetylcholine receptor expression, and neurotrophic support.

A wide range of evidence in rodents suggests glucose enhances memory processes by increasing the synthesis and release of the neurotransmitter acetylcholine. Glucose may enhance acetylcholine by providing energy or substrates for a number of cellular processes, including production of acetyl-CoA, sodium-dependent choline uptake, and transport of acetylcholine into vesicles. Glucose may also enhance acetylcholine release independent of acetylcholine synthesis by modulating stimulation-

secretion coupling in neurons. Early evidence that glucose may enhance acetylcholine synthesis came from studies showing that peripheral glucose administration could increase high-affinity choline uptake in the hippocampus of mice (Micheau et al., 1995; Messier et al., 1990). Other studies utilized *in vivo* microdialysis techniques in rats to directly measure acetylcholine release following glucose administration. Several of these microdialysis studies found that glucose could potentiate enhancements or attenuate reductions in acetylcholine release produced by pharmacological agents, such as scopolamine, morphine, and muscimol (Degroot et al., 2003; Durkin et al., 1992; Ragozzino et al., 1994b).

A number of microdialysis studies monitored the effects of glucose on acetylcholine release while rodents were engaged in behavioral memory tasks. In a study by Kopf et al. (2001), the authors found that low intraperitoneal doses of glucose plus choline in mice acted synergistically to improve retention latencies for inhibitory avoidance, while enhancing hippocampal acetylcholine release. Ragozzino et al. performed a series of microdialysis studies in rats utilizing the spontaneous alternation working memory task. Ragozzino and Gold (1995) found that concomitant injections of glucose with intraseptal morphine reversed morphine-induced deficits in acetylcholine release and working memory. Later, Ragozzino et al. (1996, 1998) showed that both intraperitoneal and direct intrahippocampal injections of glucose enhanced hippocampal acetylcholine release while improving spontaneous alternation performance. Interestingly, Ragozzino et al. (1998) found that unilateral injections of glucose into the hippocampus enhanced acetylcholine release both ipsalateral and contralateral to the injection site, suggesting that glucose may promote communication between

hippocampal hemispheres through feedback to the medial septum or by some other mechanism.

Importantly, glucose enhances acetylcholine release only under conditions of training or pharmacological manipulation, but not while animals are at rest, indicating glucose is a modulator of acetylcholine activity (Degroot et al., 2003; Durkin et al., 1992; Kopf et al., 2001; Messier et al., 1990; Ragozzino et al., 1996, 1998). Thus, there are likely regional variations in glucose-mediated acetylcholine release, depending on task demands, which may promote signaling through specific acetylcholine receptor types. To examine this possibility, a number of behavioral studies have administered glucose peripherally in combination with an antagonist for muscarinic or nicotinic receptors, which are the two major acetylcholine receptor types in the brain. Glucose attenuated or completely reversed antagonist-induced behavioral deficits in a variety of tasks, including spontaneous alternation, inhibitory avoidance, radial arm maze, and exploration of a novel environment (Blanchard and Duncan, 1997; Kopf and Baratti, 1994; Ragozzino and Gold, 1991; Ragozzino et al., 1994a; Stone et al., 1988, 1991, 1995). In one case, glucose reversed deficits in the spontaneous alternation task following co-administration of subthreshold doses of a nicotinic and muscarinic antagonist (Ragozzino et al., 1994a). Although the results of these studies suggest glucose does not work through any specific acetylcholine receptor type, the findings are open to a large number of interpretations. For example, consider the case in which glucose reversed spontaneous alternation deficits caused by mecamylamine, a general nicotinic receptor antagonist (Ragozzino and Gold, 1991). In this case, glucose may have improved task performance by enhancing acetylcholine release, which (a)

activated unblocked muscarinic receptors; (b) outcompeted mecamylamine for nicotinic binding sites; and/or (c) activated nicotinic receptors that have a low affinity for mecamylamine. Glucose may have also enhanced the release of other neurotransmitters or facilitated other memory processes. In addition, mecamylamine is known to affect a variety of processes not specific to memory, such as attention and locomotion. Glucose may have improved behavioral performance by reversing these non-specific effects of mecamylamine. Thus, based on these experiments alone, it is difficult to determine if glucose may be promoting signaling through any specific acetylcholine receptor type.

The established link between glucose and acetylcholine in modulating memory processes suggests age-related deficits in glucose availability may inhibit acetylcholine synthesis and release. These acetylcholine deficits may impair signaling processes through muscarinic and nicotinic receptors, thus causing or contributing to age-related memory impairments. Indeed, a major reason cholinergic neurons are more susceptible to pathological aging processes may be because of their high dependence on glucose metabolism for both energy production and synthesis of acetyl-CoA, a key substrate in acetylcholine synthesis (Hoyer, 2000; Meier-Ruge et al., 1994).

1.4. ACETYLCHOLINE RECEPTORS IN AGING AND MEMORY PROCESSES

Acetylcholine activates both muscarinic and nicotinic signaling processes in the brain. Muscarinic acetylcholine receptors are G protein-coupled receptors widely distributed throughout human and rodent brains. There are five major subtypes of muscarinic receptors in the brain, designated M_1 through M_5. M_1 receptors are the most abundant subtype. M_1 receptors also have the highest expression in brain regions that

exhibit known age- or pathology-related reductions in cholinergic function or innervation (Ehlert et al., 1995; Levey et al., 1991; Wevers, 2011). In addition, M_1 receptors do not show major reductions or changes in distribution with aging or age-related pathologies. Thus, M_1 receptors have become a target for enhancing cognition in Alzheimer's disease and age-related memory decline. However, the efficacy of M_1 receptor agonists is complicated by a large number of peripheral side effects (Ehlert et al. 1995; Terry et al. 2011).

A major focus of the present work is on nicotinic acetylcholine receptors, which play an important role in learning and memory processes and are one of the most promising current drug targets for enhancing cognition in neuropsychiatric and neurological disorders (Cincotta et al., 2008; Kenney and Gould, 2008; Levin et al., 2006; Picciotto and Zoli, 2002; Terry et al. 2011). Neuronal nicotinic receptors are ionotropic receptors that are found both pre- and post-synaptically. At pre-synaptic sites, nicotinic receptors regulate the release of neurotransmitters, such as GABA, glutamate, and norepinephrine. Post-synaptically, nicotinic receptors function in membrane depolarization and activation of intracellular signaling cascades. Nicotinic receptors are composed of various combinations of five membrane-spanning subunits. Nine α (α2–α10) and three β (β2–β4) subunits have been identified, making a large number of receptor subtype combinations possible. However, the heteromeric α4β2 and homomeric α7 receptor subtypes have garnered the most attention due to their high levels of expression in the brain and roles in a variety of learning and memory processes. α4β2 receptors are very widely distributed throughout the brain, whereas α7 receptors are more regionally localized to the cortex, hypothalamus, and limbic regions,

such as the hippocampus and amygdala (Dominguez del Toro et al., 1994; Gotti and Clementi, 2004; Wevers, 2011).

Nicotinic α4β2 and α7 receptors are important in a variety of learning and memory processes. Both α4β2 and α7-mediated signaling appear to play crucial roles in spatial working memory, as assessed mainly by the radial arm maze task in rodents. Infusions of either methyllycaconitine (MLA) or dihydrobetaerythroidine (DHβE), which are competitive antagonists for α7 and α4β2 receptors, respectively, into the ventral hippocampus, dorsal hippocampus, amygdala, or frontal cortex, impaired working memory in the radial arm maze (Addy et al., 2003; Bettany and Levin, 2001; Chan et al., 2007; Levin et al., 2002; Nott and Levin, 2006). Likewise, various agonists for α7 and α4β2 receptors improved working memory performance in the radial arm maze (Chan et al., 2007; Levin et al., 1999). A more recent study found that mice with knockouts of the β2 or α7 subunits had significant deficits in radial arm maze testing, with some variations by sex (Levin et al., 2009). Although α4β2 and α7 receptors appear to function very similarly in the radial arm maze task, there are also some functional differences between these receptor subtypes. For example, there are a number of studies indicating a specific role for α4β2 but not α7 receptors in mediating the effects of nicotine on enhancing memory for contextual fear conditioning (Davis and Gould, 2006, 2007; Davis et al., 2007; Kenney et al., 2012). Conversely, there is more evidence indicating a role for α7 compared to α4β2 receptors in spatial learning and memory as assessed in the water maze (Curzon et al., 2006; Meyer et al., 1997; Ren et al., 2007; Timmermann et al., 2007; Vicens et al., 2011), although direct comparisons with α4β2 receptors are often lacking in these studies.

An unusual feature of α7 receptors is their particularly high permeability for calcium. Although α4β2 receptors are moderately permeable to calcium, differences in calcium permeability between α7 and α4β2 receptors may help explain some of their functional differences. For example, α4β2 and α7 receptors have significantly different and often opposing roles in regulating synaptic plasticity, especially LTP. A large number of studies indicate that activation of α7 receptors facilitates the induction and maintenance of hippocampal LTP (e.g. Biton et al., 2007; Hunter et al., 1994; Kroker et al., 2011; Lagostena et al., 2008; Matsuyama and Matsumoto, 2003; Matsuyama et al., 2000; Ondrejcak et al., 2011; Söderman et al., 2011). In contrast, activation of α4β2 receptors appears to impair the generation and have no effect on the maintenance of hippocampal LTP (Kroker et al., 2011; Mao et al., 2011; Wang et al., 2006; Wu et al., 2008). Differences in calcium permeability may also help explain differences in the ability of α4β2 and α7 receptors to activate downstream molecular signaling pathways, which is discussed in more detail later.

Like with muscarinic receptors, reduced cholinergic function or innervation in aging and age-related pathologies may inhibit signaling through nicotinic receptors, providing a potential therapeutic target. Unlike with muscarinic receptors, there is a general consensus that nicotinic receptors and their subunits slowly decline in numbers during aging, with some regional specificity. In Alzheimer's patients, a significant reduction in nicotinic receptors in specific brain areas, such as the parahippocampal region, remains one of the most consistent markers of the disease (Gotti and Clementi, 2004; Picciotto and Zoli, 2002; Schliebs and Arendt, 2011). In addition, neurons expressing high levels of nicotinic receptors, especially of the α7 subtype, may be

particularly vulnerable to degeneration in Alzheimer's disease (D'Andrea and Nagele, 2006).

The α7 and α4β2 subtypes have been the major nicotinic receptor targets for enhancing cognition in aging and Alzheimer's disease. However, much of the current attention is on α7 receptors agonists, which have yielded some promising results in clinical trials (Terry et al., 2011). The focus on α7 receptors may be due to their confined distribution in the brain, limiting the potential side effects of α7 agonists. In addition, unlike α4β2 receptors, α7 receptors do not play a major role in the addictive properties of nicotine (De Biasi and Dani, 2011), meaning there is less potential for abuse of drugs targeting α7 receptors. Pre-clinical studies suggest nicotinic agonists may compete with β-amyloid peptides for α7 receptor binding sites, promoting cell survival or cell death, respectively. This hypothesis may provide a crucial link to understanding the pathoetiology of Alzheimer's disease (Buckingham et al., 2009; Jürgensen and Ferreira, 2010; Parri et al., 2011).

1.5. (ALMOST) EVERYBODY LOVES CREB

Acetylcholine-related signaling activates a variety of downstream molecular memory pathways. Particularly relevant to the present studies, nicotinic agonists mediate the calcium-dependent activation of cAMP response element binding protein (CREB) in the hippocampus and other brain areas. CREB is widely implicated in the formation of long-lasting memories, and may provide an important downstream marker of cholinergic signaling processes related to memory.

CREB is a nuclear protein that is part of a family of transcription factors containing a conserved basic region-leucine zipper (bZIP) domain. CREB is activated

mainly by phosphorylation, which can be triggered by a variety of intracellular signaling processes. Phosphorylation at serine 133 is considered critical for CREB activation (Montminy et al., 1990). Once activated, CREB binds to regulatory sequences of DNA as a homodimer or as a heterodimer with another bZIP-family transcription factor, thus altering expression of a large number of downstream genes. The specific set of genes regulated by CREB is determined by many factors, including the cell type, site(s) of phosphorylation, dimerization properties, and interactions with co-activators and other regulatory proteins (Shaywitz and Greenberg, 1999; Alberini, 2009).

CREB is a ubiquitous protein involved in a wide variety of brain processes, including cell survival, neurogenesis, addiction, depression, synaptic plasticity, and memory (Alberini, 2009; Blendy, 2006; Carlezon et al., 2005; Merz et al., 2011; Persengiev and Green, 2003; Robison and Nestler, 2011). However, neuronal CREB is best known for its role in memory processes, where it is believed to initiate changes in gene expression that convert short-term memory and short-term alterations in synaptic plasticity to more permanent and durable forms (Alberini, 2009; Benito and Barco, 2010; Carlezon et al., 2005; Lee et al., 2008; Silva et al., 1998; Yin and Tully, 1996). Electrophysiological studies in *Aplysia* and studies of olfactory memory in *Drosophila* provided early evidence that CREB may play an evolutionarily conserved role in memory formation. Soon after, the two major isoforms of CREB with the highest expression in the brain were genetically knocked out in mice. These CREB mutant mice were surprisingly viable given the involvement of CREB in development. However, they had deficits in a variety of hippocampal-dependent memory tasks, including contextual fear conditioning, water maze, and social transmission of food preference

(Bourtchuladze et al., 1994; Kogan et al., 1997). These effects on memory were generally limited to long but not short training-testing intervals (although see discussion below).

The early CREB studies provided the groundwork for numerous other investigations of CREB function in memory and synaptic plasticity. An abundance of findings indicate that blocking CREB function in specific brain areas via mutations, pharmacological inhibitors, or RNA interference, impairs memory when tested at long but not short intervals after training (Brightwell et al., 2005, 2008; Florian et al., 2006; Guzowski and McGaugh, 1997; Kogan et al., 2000; Lamprecht et al., 1997; Warburton et al., 2005; Zhang et al., 2003). Conversely, upregulating CREB function improves memory in a number of tasks (Brightwell et al., 2007; Josselyn et al., 2001; Sekeres et al., 2010; Suzuki et al., 2011). These behavioral studies are analagous to many electrophysiological studies suggesting that CREB is involved in the late but not early phase of LTP (Barco et al., 2002; Bourtchuladze et al., 1994; Davis et al., 2000; Jancic et al., 2009).

A number of behavioral studies have shown that CREB phosphorylation at serine 133 increases in a brain region-specific and task-dependent manner after training. Some of these studies indicate specific time windows of CREB phosphorylation following training (Bernabeu et al., 1997; McLean et al., 1999; O'Connell et al., 2000). For example, Bernabeu et al. (1997) trained rats on a step-down inhibitory avoidance task and analyzed phosphorylated CREB (pCREB) expression in hippocampal area CA1. They found that pCREB levels increased at 0, 3, and 6 hours, but not at 0.5 or 9 hours after training, suggesting a biphasic pattern of CREB activation. In contrast,

Taubenfeld et al. (1999, 2001) has shown an immediate and sustained activation of pCREB following inhibitory avoidance training, which persists for up to 20 hours. Clearly, differences in training parameters may have a profound impact on the temporal expression profile of pCREB activation.

A variety of neurotransmitter- and growth factor-initiated signaling processes can lead to CREB activation. However, the focus here is on cholinergic mechanisms of CREB phosphorylation. Acetylcholine can signal through M_3 muscarinic receptors to phosphorylate CREB, which promotes cell survival in neuroblastoma and other nerve cells (Greenwood and Dragunow, 2002, 2010). Acetylcholine can also phosphorylate CREB through nicotinic receptor signaling. Nicotine administration activates calcium-dependent CREB phosphorylation in a variety of neuronal cell types in both *in vitro* and *in vivo* models (Chang and Berg, 2001; Hu et al., 2002; Nakayama et al., 2001; Pascual et al., 2009; Tang et al., 1998). This process is mediated by an ERK/MAPK pathway and is modulated by L-type voltage-dependent calcium channels. Further, nicotine-induced CREB phosphorylation occurs through both α7 and non-α7 receptor subtypes. Several studies have found that specific α7 receptor agonists enhanced memory in a battery of cognitive tests, which correlated with increased ERK1/2 and pCREB activation in the brain (Bitner et al., 2007, 2010; Tietje et al., 2008).

Although a predominantly stated view is that CREB-mediated transcription is required and in some cases sufficient for the formation of long-term memory and LTP, not everyone agrees with this interpretation. This is mainly because there are a number of studies in CREB knockout and mutant mice that contradict this hypothesis. Balschun et al. (2003) found that mice with complete deletions in all CREB isoforms in the

hippocampus showed only slight impairments in the water maze and no impairments in contextual fear conditioning, and had no deficits in the formation or maintenance of LTP in hippocampal area CA1. Gass et al. (1998) similarly found that two different CREB mutant strains had no memory-related deficits in the water maze or in a social transmission of food preference task, and had normal LTP in the dentate gyrus and area CA1 of the hippocampus. Rammes et al. (2000) found only mild impairments in conditioned fear and no effects on LTP in the basolateral amygdala of mice expressing a dominant negative form of CREB mutated at Ser133. Although, as discussed, several studies do indicate deficits in long-lasting memory in CREB mutant mice, these deficits can often be reversed by altering training procedures, administering memory-enhancing agents, or using a different hybrid background (Frankland et al., 2004; Graves et al., 2002; Kogan et al., 1997). There are also examples of CREB mutations altering what would generally be considered short-term memory processes (Graves et al., 2002; Suzuki et al., 2011). Moreover, other studies indicate that specific inhibitors of CREB function, such as antisense oligonucleotides, or more general transcription or protein synthesis inhibitors, may impair memory via non-specific effects on neurotransmitters or other memory modulators (Canal et al. 2007, 2008; Qi and Gold, 2009; Sadowski et al., 2011). Therefore, it is important to keep in mind that multiple parallel signaling mechanisms can support the formation of long-lasting memories and, if CREB is important for memory formation, the parallel mechanisms may compensate for memory deficits related to CREB dysfunction.

1.6. CREB, AGING, AND MEMORY

Deficits in CREB function are widely implicated in cognitive impairments occurring during aging and in age-related pathologies, such as Alzheimer's disease. Animal studies have identified significant age-related reductions in CREB function, especially regarding behaviorally-induced CREB phosphorylation at serine 133. However, these studies primarily focus on changes in hippocampal CREB, providing little evidence that CREB function is altered in other brain regions. In addition, little is known about why age-related deficits in CREB arise, including which upstream activators of CREB function may be impaired.

Investigations of hippocampal total CREB levels in old rodents are mixed, with several studies indicating reductions and others suggesting no significant changes during aging (Brightwell et al., 2004; Countryman and Gold, 2007; Foster et al., 2001; Kudo et al., 2005; Porte et al., 2008a; Trofimiuk et al., 2010). Inconsistencies among these studies may have resulted from differences in the strains of rats or mice or in how the tissues were collected and analyzed. Hippocampal pCREB levels at baseline, in the absence of any behavioral training, are generally lower in old compared to young rodents. Interestingly, age-related reductions in pCREB are region-specific and do not necessarily coincide with regional deficits in total CREB (Countryman and Gold, 2007; Kudo et al., 2005). Hattiangady et al. (2005) suggested that baseline pCREB levels in the hippocampus begin declining as early as middle age. However, one study found no reductions in baseline pCREB in areas CA1, CA3, or the dentate gyrus of aged mice (Porte et al., 2008a). Since many studies only measure pCREB responses to training or only present pCREB data normalized to total CREB levels, it is often difficult to gauge age-related differences in baseline pCREB levels.

One consistent finding is that aged animals are impaired in their ability to activate pCREB in response to behavioral or sensory stimuli (Countryman and Gold, 2007; Kudo et al., 2005; Monti et al., 2005; Porte et al., 2008a; Zhang et al., 1996; Zhao et al., 2009). Further, age-related deficits in hippocampal pCREB activation often correlate with memory impairments, as assessed in a variety of behavioral tasks. Deficits in pCREB activation occur following training in social transmission of food preference (Countryman and Gold, 2007), fear conditioning (Monti et al., 2005), step-down inhibitory avoidance (Zhao et al., 2009), and the water maze (Porte et al., 2008a; Zhao et al., 2009). A number of recent experiments have examined substances, such as green tea, ginsenoside, and St. John's wort, that may reverse age-related deficits in pCREB in concert with memory enhancement (Li et al., 2009; Trofimiuk et al., 2010; Xu et al., 2010; Zhao et al., 2009). Although these studies do show improvements in both behavioral performance and pCREB activation in old rodents, these results are not likely specific to memory processes, since the compounds are often administered for several weeks or months prior to training. Thus, more work is needed to determine how various pharmacology-based methods of memory enhancement interact with CREB-mediated signaling processes. Using a non-pharmacological approach, Aguiar et al. (2011) examined if short bouts of exercise over five weeks in aged Wistar rats could improve memory while enhancing hippocampal markers of synaptic plasticity. They found that the exercise group, compared to age-matched controls, had better memory in a step-down inhibitory avoidance task and water maze, and improved hippocampal pCREB and BDNF expression.

A number of labs have attempted to improve CREB function during aging using transgenic mice. In a longitudinal study, Mouravlev et al. (2006) used somatic gene transfer of CREB or inducible cAMP early repressor (ICER) to modify hippocampal CREB expression in young adult rats. At 15-months of age, the CREB-transduced rats had better memory than similarly aged controls, as assessed by the Barnes circular maze and inhibitory avoidance paradigms. In contrast, the ICER-transduced rats had significant memory deficits, even more so than in the age-matched controls. Likewise, other transgenic mouse models in which CREB function is enhanced throughout much of the lifespan exhibit improved memory during aging (Fukushima et al., 2008; Xing et al., 2010). However, in contrast to these longitudinal studies, a recent experiment showed that viral-mediated overexpression of CREB into the hippocampus improved spatial memory in young adult but not middle-aged rats, suggesting that middle-aged rats may have deficits in upstream activators of CREB phosphorylation (East et al., 2011).

CREB function is dysregulated in several human age-related neurodegenerative diseases, including Alzheimer's disease and Huntington's disease. Recently, there has been a renewed interest in examining CREB dysfunction in Alzheimer's disease, with particular focus on how β-amyloid peptides may alter or disrupt CREB-mediated signaling processes. In addition, several classes of drugs that enhance CREB function, including nicotinic α7 receptor agonists are currently in development for treating cognitive impairments in Alzheimer's disease (Bitner, 2011; De Felice et al., 2007; Saura and Valero, 2011; Wang and Bibb, 2011).

1.7. OBJECTIVES

The review of the research literature in the preceding sections illuminates a potential neuroendocrine pathway by which emotional events lead to memory enhancement in young adult animals (Figure 1.1). In old animals, a deficit in peripheral glucose release early in this pathway may result in impairments in all subsequent steps, thus leading to rapid forgetting instead of memory facilitation (Figure 1.2). In particular, deficits in peripheral glucose release may lead to impairments in acetylcholine release, nicotinic acetylcholine receptor signaling, and CREB phosphorylation, thus producing age-related memory impairments. This is the overarching hypothesis guiding the present work.

The main objective of the present studies is to examine how an age-related uncoupling between peripheral epinephrine and glucose release may impair downstream memory processes, particularly those related to cholinergic signaling. The experiments in Chapter 2 examine if a dysfunction in the blood glucose response to circulating epinephrine contributes to age-related impairments in memory and hippocampal acetylcholine release. The work in Chapter 3 examines the relationship of CREB and pCREB to the formation of long-lasting memory in young and old rats. The studies in Chapter 4 attempt to determine the effects of peripheral epinephrine and glucose administration on memory and CREB activation in young and old rats. An additional goal in Chapter 4 is to determine if direct intrahippocampal administration of glucose can reverse age-related memory impairments. Finally, the experiments in Chapter 5 utilize nicotinic acetylcholine receptor antagonists in young rats to model age-related memory impairments, investigating the relationship among glucose, nicotinic acetylcholine receptors, and memory.

1.8. FIGURES

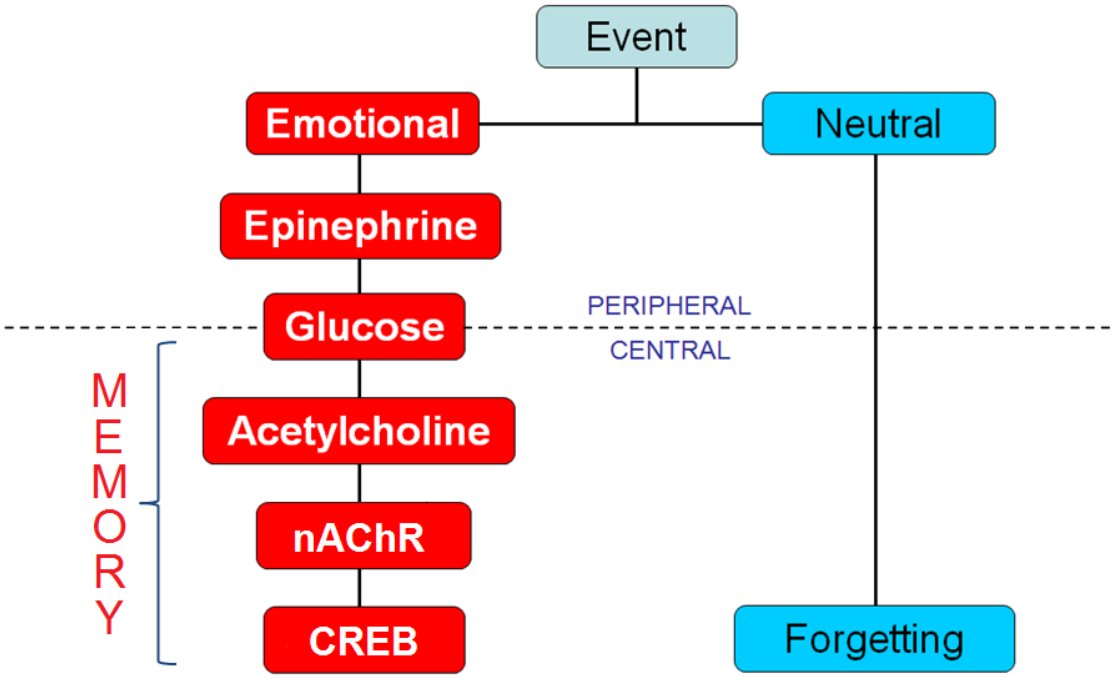

Figure 1.1. Model for memory enhancement by epinephrine.

In young adult animals, emotional or stressful events induce epinephrine release from the adrenal medulla. Epinephrine binds to hepatic adrenergic receptors and stimulates the release of glucose into the blood. Glucose enhances cholinergic signaling in the brain, activating markers of synaptic plasticity and improving memory for the events.

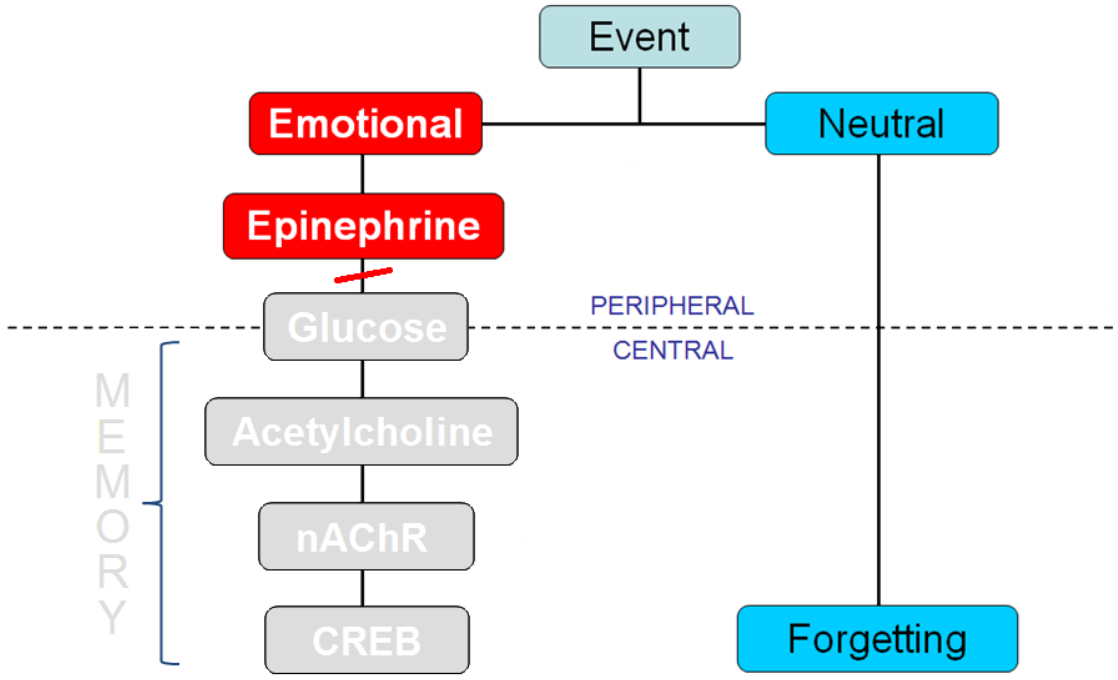

Figure 1.2. Model for age-related forgetting.

In old animals, there is an uncoupling between peripheral epinephrine and blood glucose. This uncoupling leads to impairments in downstream memory processes.

CHAPTER 2: AGE-RELATED MEMORY IMPAIRMENTS DUE TO REDUCED BLOOD GLUCOSE RESPONSES TO EPINEPHRINE[1]

Increases in blood glucose levels are an important component of the mechanisms by which epinephrine enhances memory formation. The present experiments addressed the hypothesis that a dysfunction in the blood glucose response to circulating epinephrine contributes to age-related memory impairments. Doses of epinephrine and glucagon that significantly increased blood glucose levels in young adult rats were far less effective at doing so in 2-year-old rats. In young rats, epinephrine and glucose were about equally effective in enhancing memory and in prolonging post-training release of acetylcholine in the hippocampus. However, glucose was more effective than epinephrine in enhancing both memory and acetylcholine release in aged rats. These results suggest that an uncoupling between circulating epinephrine and glucose levels in old rats may lead to an age-related reduction in the provision of glucose to the brain during training. This in turn may contribute to age-related changes in memory and neural plasticity.

2.1. INTRODUCTION

Epinephrine, released from the adrenal medulla in response to many experiences, enhances memory in both rodents and humans (Cahill and Alkire, 2003; Gold, 2001, 2005; Gold and McCarty, 1995; Gold and McGaugh, 1975; Gold and van Buskirk, 1975; Korol and Gold, 2007; McGaugh and Roozendaal, 2002). Because

[1] This chapter appeared in its entirety in the journal Neurobiology of Aging and is referred to later in this dissertation as Morris et al., 2010. Morris, K.A., Chang, Q., Mohler, E.G., and Gold, P.E. (2010). Age-related memory impairments due to reduced blood glucose responses to epinephrine. Neurobiol. Aging, 31, 2136-2145. This article is reprinted with the permission of the publisher.

epinephrine does not readily cross the blood–brain barrier, the hormone most likely enhances memory by engaging peripheral mechanisms that modify brain functions. Glucose, released from the liver in response to circulating epinephrine, enhances memory, with results similar to epinephrine (Gold, 1995, 2005; Korol and Gold, 2007; McNay and Gold, 2002; Messier, 2004). However, some important differences between the two treatments support the idea that increases in blood glucose mediate the effects of epinephrine on memory. First, peripheral adrenergic receptor antagonists block the memory-enhancing effects of peripheral epinephrine injections (Sternberg et al., 1986), but not of glucose (Gold et al., 1986), suggesting that glucose bypasses the need to activate peripheral adrenergic receptors to enhance memory. Second, glucose enhances memory in food-deprived rats but epinephrine does not (Talley et al., 2000), which is consistent with the diminished increases in blood glucose to epinephrine injections seen after food deprivation. Additionally, microinjections of glucose into the lateral ventricles, medial septum, hippocampus, and amygdala enhance memory (Canal et al., 2005; Krebs and Parent, 2005; Pych et al., 2006; Ragozzino et al., 1996, 1998; Schroeder and Packard, 2003). These findings are consistent with the idea that glucose acts directly on the brain to modulate memory processing (Gold, 2005, 2008; Korol and Gold, 2007).

There are several mechanisms by which glucose might modulate brain functions, including regulating neural excitability and stimulus–secretion coupling by closing potassium-ATP channels (Rashidi-Pour, 2001; Stefani and Gold, 2001; Stefani et al., 1999) and regulating cell signaling mechanisms such as the mammalian target of rapamycin (TSC-mTOR) cascade (Dash et al., 2006). Additionally, both systemic and

central injections of glucose appear to act on memory through cholinergic mechanisms (Durkin et al., 1992; Gold, 1995; Kopf et al., 2001). In particular, *in vivo* microdialysis studies show that glucose augments training-related release of acetylcholine (ACh) in a manner related to enhancement of memory (cf. McNay and Gold, 2002; Ragozzino et al., 1996, 1998). Together with the relationships between loss of cholinergic functions and aging (cf. Bartus et al., 1982; Mesulam, 2004), these findings suggest a possible link between glucose and ACh during aging. The present experiment adds support to these relationships.

Regional fluctuations in brain glucose concentrations in extracellular fluid (ECF) are evident during memory testing. ECF glucose levels in the hippocampus decrease substantially while rats are tested on a spatial working memory task (McNay and Gold, 2001; McNay et al., 2000). Systemic injections of glucose block this decrease when enhancing memory, suggesting that ECF glucose levels limit the efficacy of memory processing. The training-induced decreases in hippocampal ECF glucose are exaggerated in aged rats. Systemic administration of glucose blocks this decrease in ECF glucose and enhances memory, raising the scores of old rats to those of young rats. Blood glucose responses to behavioral testing are decreased in senescent rats, but circulating epinephrine responses to training or stress are actually increased (Gold, 2005; Mabry et al., 1995a,b,c). The increase in release of epinephrine without subsequent increases in circulating glucose levels suggests a breakdown in a neuroendocrine pathway important for modulating memory in old rats, potentially at the step in which glucose release from the liver is coupled to the binding of epinephrine. The uncoupling between peripheral epinephrine and glucose release may reduce the

amount of glucose available to the brains of old rats during memory tasks and lead to the memory impairments and other cognitive changes seen in aged rats, mice and humans (Buckner, 2004; Chawla and Barnes, 2007; Disterhoft and Oh, 2006; Gazzaley and D'Esposito, 2007; Gold, 2005; Korol, 2002; Mattson et al., 2004). The present experiments examine the role of uncoupled epinephrine–glucose responses in modulation of memory and in augmenting training-related release of ACh in aged rats.

2.2. MATERIALS AND METHODS

2.2.1. Subjects

Young adult (3–5 months) and old (24–26 months) male Fischer-344 rats from NIA colonies were individually housed in translucent cages with a 12-h light/dark cycle (lights on at 07:00 h) and *ad libitum* access to food and water. One set of rats was used for the blood glucose measurements (Figs. 2.2 and 2.3). A second set of rats was used for the memory and neurochemistry experiments (Figs. 2.4–2.6).

2.2.2. Blood glucose measurements

Rats were handled for 4–5 min each day for 5 consecutive days prior to blood glucose measurements. Epinephrine (0.1 mg/kg [Ns = 6 young, 5 old] or 0.3 mg/kg [Ns = 6 young, 4 old]) or glucagon (200 µg/kg or 400 µg/kg [all Ns = 4]) was injected subcutaneously, followed by monitoring of blood glucose levels by collecting blood drops from the tip of the tail with a Penlet® (Lifescan, Inc., Milpitas, CA) and measuring their glucose levels with a One Touch® glucometer (Lifescan, Inc., Milpitas, CA). Data were discarded for three old rats receiving epinephrine injections because their baseline blood glucose levels were markedly low.

2.2.3. Surgery

Rats were anesthetized with isoflurane and placed in a stereotaxic apparatus with skulls in a horizontal orientation. A CMA/11 guide cannula (CMA, North Chelmsford, MA) was implanted above the central portion of the ventral hippocampus in both young [coordinates: −5.5 mm from bregma; ±4.8 mm lateral; −4.2 mm deep from skull] and old [coordinates: −5.8 mm from bregma; ±5.0 mm lateral; −4.8 mm deep from skull] rats. Skull screws were inserted and the entire assembly was anchored in place with dental cement. Beginning 1 week after surgery, rats were handled 4–5 min each day for 5 consecutive days prior to microdialysis and behavioral training.

2.2.4. Microdialysis

On the day of training, a 4-mm CMA/11 microdialysis probe (CMA) was inserted into the ventral hippocampus. Brains were perfused continuously at a rate of 0.67 µl/min with artificial cerebral spinal fluid (aCSF: 128 mM NaCl, 2.5 mM KCl, 1.3 mM CaCl2, 2.1 mM MgCl2, 0.9 mM NaH2PO4, 2.0 mM Na2HPO4, 1.0 mM dextrose, pH 7.4) containing 200 nM of the acetylcholinesterase inhibitor neostigmine (Chang et al., 2006). The 1.0 mM dextrose in the aCSF was based on previous work showing that extracellular glucose levels in the hippocampus of awake young and aged rats are about 1.0 mM (McNay and Gold, 1999). Samples collected during the first hour of microdialysis were discarded to allow for baseline stabilization (Westerink and Timmerman, 1999). Ten total samples were collected in 10-min intervals immediately prior (samples B1–4), during (P1), and after (P2–6) training. The final volume of each sample was 6.7 µL.

2.2.5. Training

After 4 baseline samples (10 min each) were collected, rats were trained on a one-trial inhibitory avoidance task. The apparatus was a trough-shaped alleyway (91 cm long, 22.9 cm wide at the top, 7.6 cm wide at the bottom, and 15.2 cm deep) divided into lit (31 cm) and dark (60 cm) compartments by a sliding door that could be lowered through the floor. Rats were placed in the lit chamber and the door was lowered. Upon entering the dark chamber, the door was closed and the rats received a brief mild footshock (0.2 mA, 0.4 s). To facilitate comparisons of drug enhancement of memory at both ages, the shock level and training conditions were selected to match 48-h memory across ages. Past evidence indicates that age-related differences in forgetting would likely be seen at longer training-test intervals (Gold et al., 1982), but this was not assessed here. Immediately after training, rats received a subcutaneous injection of saline (0.9%) (Ns = 18 young, 15 old), epinephrine (0.1 mg/kg) (Ns = 8 young, 8 old), or glucose (250 mg/kg) (Ns = 9 young, 7 old). The rats were then returned to the holding cage as microdialysis continued. Memory, measured as the latency to enter the dark compartment (maximum = 300 s), was assessed 48 h later.

2.2.6. HPLC

Microdialysis samples were assayed for ACh using high performance liquid chromatography (HPLC) with electrochemical detection (BAS; Bioanalytical Systems, West Lafayette, IN). Five µl of each sample were manually injected into the system via an injection valve with a 10 µl loop (Rheodyne, Rohnert Park, CA). Samples were separated using an ion-exchange microbore analytical column (BAS P/N MF 8904, 530 mm×1 mm) followed by a microbore ACh/choline immobilized enzyme reactor containing acetylcholinesterase and choline oxidase (BAS P/N MF-8903, 50 mm×1

mm). Electrochemical detection was performed by a 3 mm glassy fiber working electrode (BASP/NMF1095) coated with a redox polymer film containing horseradish peroxidase and an auxiliary electrode with a radial flow electrochemical thin-layer cell and a 13 mm thin-layer gasket (BAS P/N MF 1091). The working electrode held a 100 mV potential relative to an Ag/AgCl reference electrode (BAS P/N MF 2078). A Shimadzu LC-10ADvp pump (Tokyo, Japan) with microstep plunger maintained the flow rate at 140 µL/min. The mobile phase contained 50 mM Na2HPO4 and 0.005% ProClin (BAS P/N CF-2150) and was adjusted to a pH of 8.5. Assays were completed within 12.5 min. The detection limit of this system was approximately 5 fmol.

2.2.7. Histology

Rats were deeply anesthetized with sodium pentobarbital prior to decapitation. Brains were removed and placed into 4% paraformaldehyde in 0.1M phosphate buffer for at least 24 h. The brains were transferred to 20% glycerol in 0.1M PB for at least 24 h. Frozen sections (50 µM) were collected with a Leica 1800 cryostat. Sections containing the guide cannulae tracts were mounted on slides, dried, stained with cresyl violet, and visualized under a microscope (Fig. 2.1). Behavioral and chemical data were discarded for those rats (N= 3) with probe sites outside of the ventral hippocampus.

2.2.8. Data analyses

All analyses were performed using Statview software. Blood glucose results and HPLC data were analyzed using repeated measures ANOVA with post hoc Fisher PLSD and t-tests where appropriate. Behavioral results were analyzed using unpaired t-

tests. Absolute levels of ACh release were calculated using recovery and internal HPLC standards.

2.3. RESULTS

2.3.1. Epinephrine and glucagon injections raise blood glucose levels less in old than in young rats

Fig. 2.2 shows the levels of blood glucose following subcutaneous injections of two different doses of epinephrine. Injections of 0.1 mg/kg epinephrine, a dose previously shown to enhance memory in young rats (Gold and van Buskirk, 1978a), produced significant increases in blood glucose levels in young rats ($F_{(1,6)} = 17.11$, $P < 0.0001$), with peak blood glucose levels increasing by 96% above baseline at 1 h after injection. In contrast, the same dose of epinephrine resulted in very modest but statistically significant ($F_{(1,6)} = 3.54$, $P < 0.05$) increases in blood glucose levels in old rats, with peak increases of only 21% above baseline evident at 30 min after injection. The difference in the increases in blood glucose levels in young vs. old rats was statistically significant ($F_{(1,6)} = 5.62$, $P < 0.05$).

In young rats, injection of a higher dose (0.3 mg/kg) of epinephrine resulted in a significant increase in blood glucose levels ($F_{(1,6)} = 24.18$, $P < 0.0001$) that was comparable to that seen at the lower dose ($F_{(1,6)} = 0.17$, $P > 0.5$). Peak blood glucose levels were evident at 1 h after injection, reaching 99% above baseline. In contrast to the results in young rats, the epinephrine-induced increases in blood glucose levels were dose-dependent in old rats. Injection of the higher dose (0.3 mg/kg) of epinephrine in old rats resulted in significant increases in blood glucose levels compared to the lower dose ($F_{(1,6)} = 7.78$, $P < 0.05$), with peak increases of 49% above baseline evident

at 1 h after injection. The difference in the increases in blood glucose levels in young vs. old rats at the higher dose of epinephrine was not significant ($F_{(1,6)} = 1.47$, $P > 0.1$).

Glucagon injections in young and old rats resulted in similar but more rapid increases in blood glucose levels compared to epinephrine, with peak levels evident at 15 min after injection (Fig. 2.3). At both doses and both ages, glucagon injections resulted in significant increases in blood glucose levels ($F_{(1,3)} = 16.53–29.34$, $Ps < 0.01$). Similar to epinephrine, there was a significant effect of age at a low (200 µg/kg) but not a high (400 µg/kg) dose of glucagon ($F_{(1,3)} = 140.81$, $P < 0.0001$ for low dose; $F_{(1,3)} = 3.35$, $P > 0.1$ for high dose). Unlike epinephrine, there was a significant effect of treatment in young but not old rats ($F_{(1,3)} = 14.34$, $P < 0.01$ in young; $F_{(1,3)} = 1.87$, $P > 0.1$ in old).

2.3.2. Glucose is more effective than epinephrine at enhancing memory in old rats

As shown in Fig. 2.4, post-training injections of either epinephrine or glucose significantly increased 48-h test latencies in the inhibitory avoidance task in young rats (t-tests: $Ps < 0.05$ for epinephrine and for glucose vs. saline). The difference in test latencies in rats injected with glucose and epinephrine did not differ significantly ($P > 0.05$). Although both epinephrine and glucose resulted in higher memory scores in young rats, only glucose significantly increased latencies on the memory tests in old rats. In old rats receiving post-training epinephrine injections, the median latency on the test trials increased from 74 s in the saline controls to 159 s in the epinephrine-treated rats, but these latencies did not differ significantly from each other ($P > 0.1$). In contrast, old rats in the glucose group had median retention latencies at the maximum of 300 s, which are scores significantly higher than those of the saline controls ($P < 0.05$). There

was no significant difference in test latencies between glucose and epinephrine-treated old rats or between glucose-treated old rats and either epinephrine- or glucose-treated young rats (Ps > 0.2).

2.3.3. Glucose modulates training-related release of ACh similarly in young and old rats, but epinephrine is more effective in young than in old rats

Fig. 2.5 shows the ACh results obtained with *in vivo* microdialysis in young and old rats. The baseline values shown are percent change from the mean baseline levels, which were obtained for each rat by averaging the 4 samples (20 min each) collected prior to training. Thus, the data are presented as percent change from baseline levels in 10-min intervals immediately prior to (B1–4 = Baseline), during (P1), and after (P2–6) training. In both young and old rats, training in the saline-treated rats resulted in increases from baseline ACh release of ~100–150%, which gradually returned to baseline in the 40 min or so after training. In young rats, there was a significant effect of treatment ($F_{(2,9)} = 3.66$, $P < 0.05$), in which post-training administration of epinephrine or glucose significantly enhanced ACh release (Fisher's PLSD: Ps < 0.05 vs. saline).

As was seen in young rats, there was a significant effect of treatment on ACh release ($F_{(2,9)} = 6.76$, $P < 0.01$) in old rats, with glucose significantly enhancing ACh release compared to saline ($P < 0.0001$). However, unlike in young rats, epinephrine did not significantly increase ACh release in old rats ($P > 0.05$), although it did result in modest non-significant increases in the duration of ACh release. These results parallel those shown in Figs. 2.2 and 2.4, in which epinephrine was deficient in increasing blood glucose levels and in enhancing memory in old rats, respectively.

2.3.4. Baseline ACh release in the ventral hippocampus is lower in old than in young rats

While the percent changes in ACh release immediately after training are similar in young and old rats, as in Fig. 2.5, there are substantial age-related differences in baseline and peak levels of ACh concentrations in the dialysates. Fig. 2.6 shows the absolute baseline and training-induced levels of ACh release in the ventral hippocampus of young and old rats. Baseline levels were significantly lower in old rats than in young rats, with values about half those seen in young rats (t-test: $P < 0.0001$). Training-related levels of ACh release were reduced in old compared to young rats with post-training injections of both epinephrine and glucose (Fisher's PLSD: $Ps < 0.05$). Thus, it is clear that the increases in concentration and duration of ACh release concentrations induced by glucose administered to aged rats bring the levels toward those of young baseline values but do not result in full amelioration of age-related decreases in release.

2.4. DISCUSSION

These findings suggest that dysfunctions in blood glucose responses to epinephrine contribute significantly to age-related memory loss. During aging, epinephrine develops reduced efficacy in increasing blood glucose levels, enhancing memory, and augmenting training-initiated increases in release of ACh in the hippocampus.

2.4.1. Impaired blood glucose responses to epinephrine and glucagon in aged rats

Doses of epinephrine and glucagon that significantly increased blood glucose levels in young rats were far less effective at doing so in old rats, in which higher doses were needed to increase blood glucose levels. These findings are consistent with others showing that a wide variety of hormones, including insulin and ACTH, show reduced functionality with age (Fink et al., 1983; Giordano et al., 2001; Parker et al., 2000). Previous work has shown that basal levels of plasma epinephrine are similar in young and aged rats, but that aged rats exhibit higher circulating epinephrine levels after placement in a novel environment, footshock, or immersion in water (Mabry et al., 1995a,b,c, 1996). The higher responses to stimulation may reflect a futile physiological attempt to generate increases in blood glucose levels. The present results support and extend these findings by showing that old rats have substantially blunted increases in blood glucose following subcutaneous injections of epinephrine.

In old rats, the uncoupling between circulating epinephrine and glucose suggests a dysfunction in the ability of the liver to produce glucose in response to epinephrine binding. There is substantial evidence for age-related changes in hepatic adrenoreceptors, including their regulation of hepatic glucose production in both rats and humans (Ebstein et al., 1985; Graham et al., 1987; Katz et al., 1993; Podolin et al., 1996; Van Ermen et al., 1992). Blockade of peripheral adrenoreceptors blocks the effects of systemic injections of epinephrine but not glucose on blood glucose levels and on enhancement of memory (cf. Gold, 2005).

The finding that blood glucose responses to both glucagon and epinephrine were reduced in old rats suggests involvement of shared cell signaling mechanisms beyond the respective receptors. Considerable evidence demonstrates age-related dysfunctions

in cell signaling pathways involved in hepatic glucose production, including activity of hepatic adenylate cyclase, involved in both epinephrine and glucagon-mediated signal transduction (Ebstein et al., 1985; Podolin et al., 2001), and mitochondrial damage, which may also be responsible for reduced rates of gluconeogenesis in hepatocytes of old rats (Liu et al., 2002; Sastre et al., 1996).

Cross-sectional studies in humans generally show a reduction in basal and stress-associated epinephrine in older individuals (Esler et al., 1995; Kjeldsen et al., 1982; Seals and Esler, 2000). However, interpretation of these cross-sectional studies is complicated by attrition (Woodruff-Pak, 1997), a particular concern here because epinephrine levels are positively associated with hypertension and other conditions (Floras, 1992; Rand and Majewski, 1984). A relatively short-term longitudinal study of aging human subjects found that increases in urinary epinephrine levels in elderly individuals over a 3-year period were associated with declines in a variety of cognitive measures (Karlamangla et al., 2005). Based on this, the authors suggest that chronic stress may be an important predictor of cognitive decline in elderly humans. Alternatively, the elevated epinephrine levels may reflect disruption of a negative feedback mechanism due to an impaired glucose response, with cognitive impairments related to loss of glucose modulation of brain functions as seen in rats.

2.4.2. Epinephrine and glucose effects on memory in aged rats

A glucose dose that is effective in enhancing memory in young rats retains that efficacy in aged rats. However, an epinephrine dose effective in enhancing memory in young rats is not as effective in old rats. Together with the blood glucose results, there is likely an age-related shift to higher doses of epinephrine needed to enhance memory

in aged rats. Direct glucose injections may enhance memory in old rats by circumventing the functional impairment of epinephrine-induced increases in blood glucose levels.

Unlike the lower dose of epinephrine, a higher dose did significantly enhance circulating blood glucose in old rats. Given this, one might expect the higher dose would significantly enhance memory for the inhibitory avoidance task, consistent with an age-related shift in the dose–response curve for memory facilitation by epinephrine. However, in attempting tests with the higher dose in aged rats, the dose resulted in apparent discomfort at the injection site that interfered with the behavioral tests, and the tests were therefore discontinued.

2.4.3. Glucose effects on brain neurochemical responses to training

Extracellular glucose levels, and the maintenance of these levels by increases in blood glucose levels (Fellows and Boutelle, 1993; Fellows et al., 1993; Korf et al., 1993), appear to be important to memory functions. ECF glucose levels in the hippocampus decrease while rats perform a spatial working memory task; the magnitude of the decrease increases as a function of task difficulty (McNay et al., 2000). Systemic injections of glucose reverse the depletion of ECF glucose and improve memory scores. The decrease in hippocampal ECF glucose levels is exaggerated in magnitude and duration in aged rats, declining by as much as 50% during memory testing (McNay and Gold, 2001). Of interest, the more rapid recovery of ECF glucose levels in young rats is associated with a rise in blood glucose levels—a rise that does not occur in aged rats.

The effects of epinephrine–glucose uncoupling on brain functions may be exacerbated by other age-related dysfunctions in mechanisms of glucose uptake into the brain. For instance, changes with age in the efficiency and plasticity of glucose transporters may result in local reductions in glucose uptake (Messier, 2004; Messier and Teutenberg, 2005). GLUT-1 is a high-affinity glucose transporter expressed in capillary endothelial cells with a principle role in mediating the facilitated uptake of glucose through the blood–brain barrier (Maher et al., 1994). GLUT-1 is reduced in specific regions of the brain, including the hippocampus, during normal aging (Gschanes et al., 2000; Vorbrodt et al., 1999) and in Alzheimer's disease (Horwood and Davies, 1994; Kalaria and Harik, 1989; Simpson et al., 1994).

There are several mechanisms by which dynamic changes in ECF glucose might regulate memory functions (cf. McNay and Gold, 2002). One possibility is that ECF glucose may regulate neural excitability by actions at K-ATP channels. In pancreatic β-cells, glucose controls insulin release by regulating intracellular ATP/ADP ratios and closing K-ATP channels (Ashcroft, 2005; Hansen, 2006). These inwardly rectifying potassium channels are present throughout the brain and expressed at high levels in the hippocampus (Dou et al., 2003; Mourre et al., 1990, 1991), offering a potential mechanism by which glucose might regulate neural excitability and stimulus–secretion coupling. Administration of drugs that close and open K-ATP channels have effects on memory similar to those of glucose availability and depletion, respectively (Banchelli et al., 2000; Ghelardini et al., 1998; Rashidi-Pour, 2001; Stefani and Gold, 2001; Stefani et al., 1999).

Changes in glucose availability, from blood glucose or by alterations in glucose transporter functions, have effects on neurotransmitters related to memory processing. Extensive evidence suggests that alterations in the cholinergic neurotransmitter system contribute to age-related and pathological memory impairments (Müller et al., 1991; Terry and Buccafusco, 2003). The finding here that baseline levels of release of ACh in the hippocampus decline with age is consistent with past results (Feuerstein et al., 1992; Terry and Buccafusco, 2003; Wu et al., 1988). The augmentation of ACh release in aged rats by glucose did not result in full amelioration of age-related declines in release even as the enhancement of memory was as robust, or more so, in aged rats. Together, the behavioral and neurochemical findings may reflect actions of glucose on neural functions other than ACh release or up-regulation of receptor and cell signaling responses to ACh in response to the decreased levels of release.

While past reports showed that glucose treatment can augment ACh release during memory testing (Ragozzino et al., 1996, 1998), the present study is the first to demonstrate an effect of a post-training memory-enhancing treatment on neurotransmitter release after an experience. Glucose was effective at augmenting training-related increases in the duration of ACh release in both young and old rats, in concert with enhancement of memory. Epinephrine also augmented the duration of ACh release and enhanced memory in young rats but, in contrast to glucose, failed to do so significantly in aged rats.

2.5. CONCLUSIONS

The findings of these experiments support the hypothesis that age-related memory impairments may arise from disruptions in the neuroendocrine regulators that

engage central memory processes. According to this view, the release of epinephrine and the subsequent elevation in blood glucose levels provide a crucial component of a process that upregulates brain mechanisms responsible for the formation of new memories. In young rats, trivial events accompanied by small physiological responses are quickly forgotten. Salient events that induce a relatively large physiological response lead to more enduring memories (Gold and McGaugh, 1975). The results here indicate that in aged rats, many experiences may be handled as “trivial” events, due to an impaired glucose response, and are therefore forgotten more quickly. Thus, the more rapid forgetting observed in old rats may reflect a chain of events, beginning with an uncoupling of the epinephrine and glucose response as a component of dysfunctions leading to deficiencies in ECF glucose levels. In turn, this deficit may depress neural excitability and release of neurotransmitters including ACh, ultimately negatively impacting memory formation and maintenance. It will be important to extend these findings by examining the effects of treatments that enhance cognitive and other brain function during aging, including caloric restriction (e.g.: Adams et al., 2008; Fontan-Lozano et al., 2007; Patel and Finch, 2002) and exercise (e.g.: Erickson et al., 2007; van Praag et al., 2005), on central and peripheral regulation of glucose levels and utilization.

2.6. FIGURES

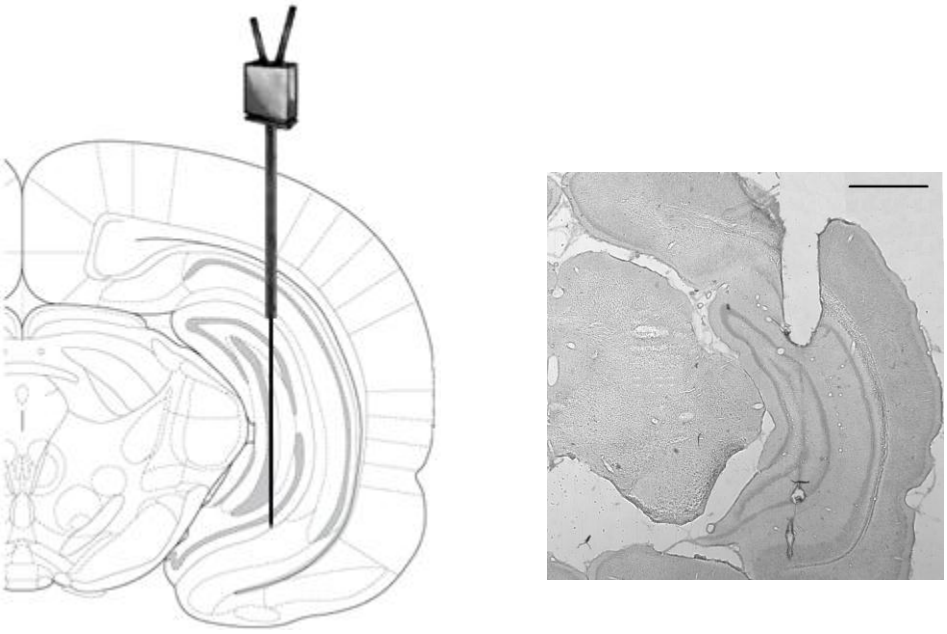

Figure 2.1. Cannula placement into the ventral hippocampus.

(Left) Illustration of the area targeted in this study, showing insertion of a CMA microdialysis probe into the ventral hippocampus (adapted from Paxinos and Watson, 2005, Figure 80). (Right) Photomicrograph of a cresyl violet-stained section showing a representative cannula placement in the ventral hippocampus of an old rat. Scale bar = 1.6 mm.

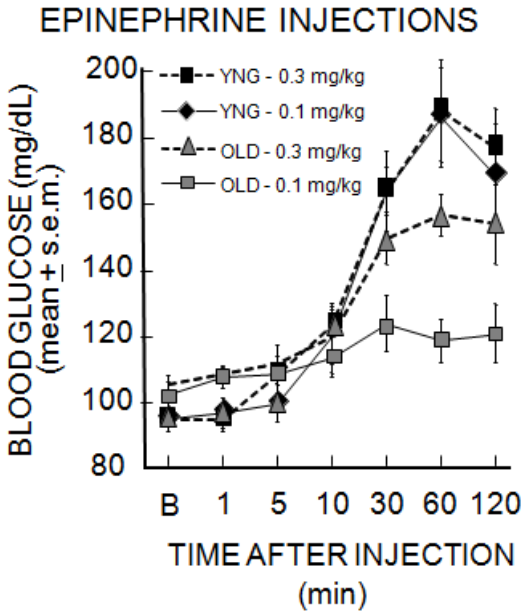

Figure 2.2. Blood glucose measurements following injections of epinephrine.

Age-related impairments in blood glucose responses were observed following a lower but not a higher dose of epinephrine.

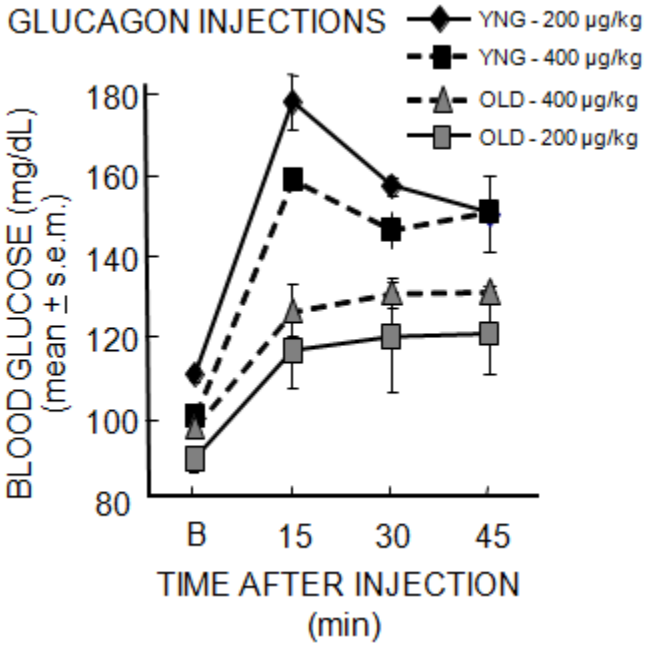

Figure 2.3. Blood glucose measurements following injections of glucagon.

Similar to epinephrine, age-related impairments in blood glucose responses were observed following a lower but not a higher dose of glucagon.

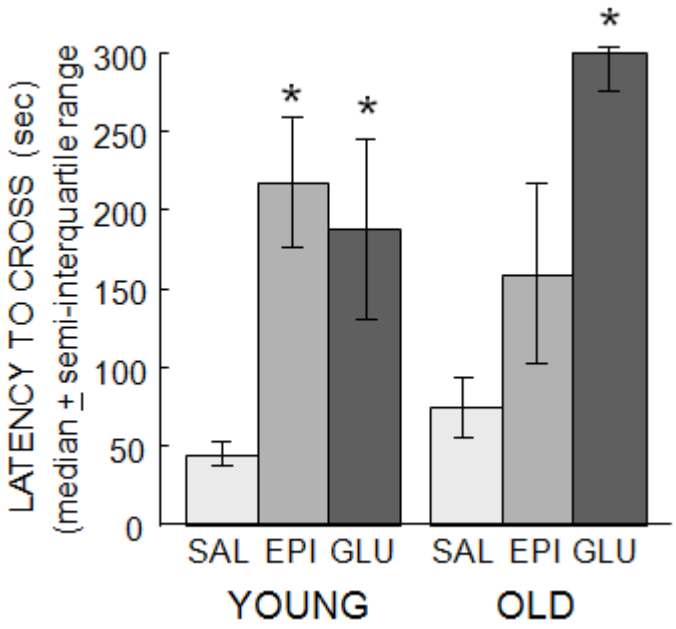

Figure 2.4. Age-related differences in epinephrine- and glucose-mediated memory enhancement.

Effects of post-training injections of epinephrine (0.1 mg/kg) and glucose (250 mg/kg) on memory tested 48 h after inhibitory avoidance training in young and old rats. Post-training injections of glucose, but not epinephrine, significantly enhanced memory in old rats. (*) *P*s < 0.05 vs. saline controls.

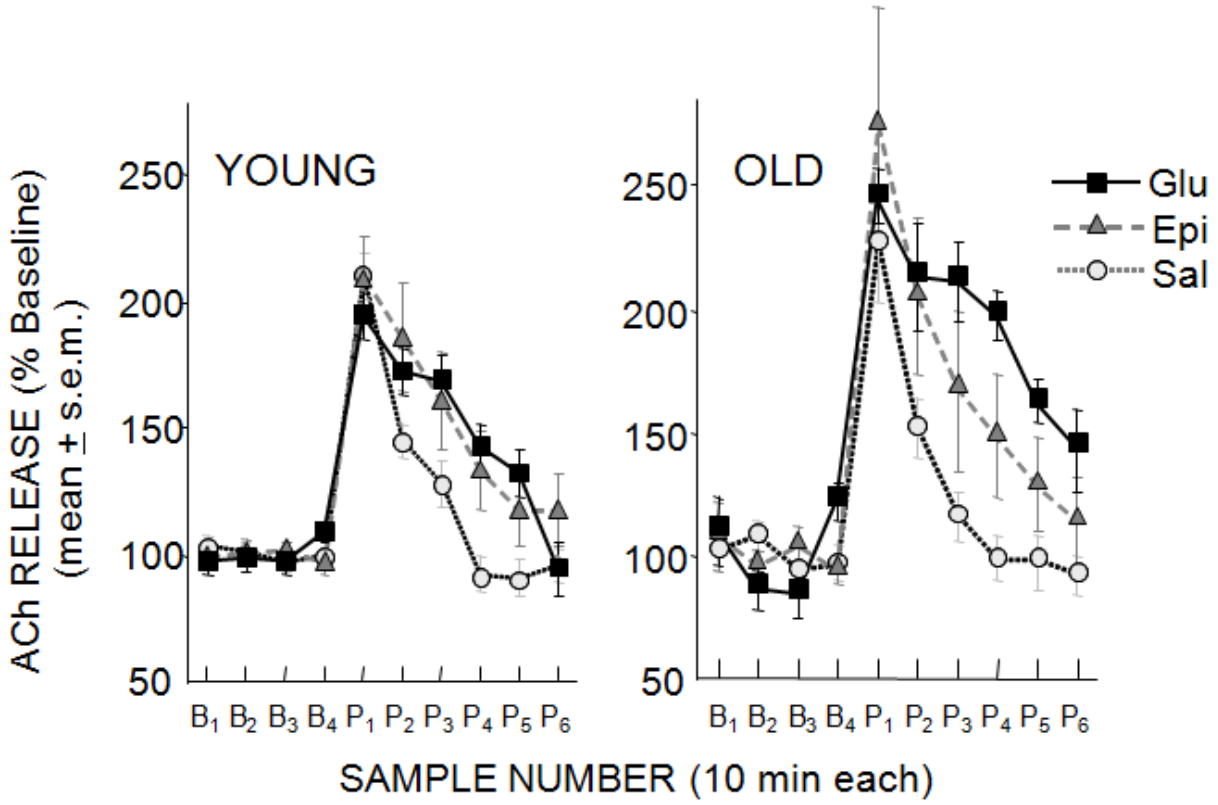

Figure 2.5. Age-related differences in modulation of acetylcholine release.

Percent changes in ACh release in the ventral hippocampus accompanying inhibitory avoidance training in young (left graph) and old (right graph) rats. Compared to saline controls, post-training injections of glucose, but not epinephrine, significantly enhanced training-related release of ACh in old rats ($P < 0.05$). B= baseline samples; P = post-training samples.

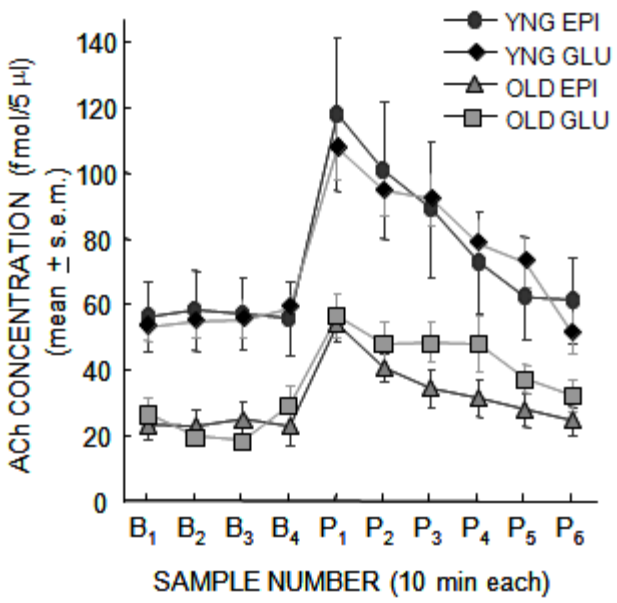

Figure 2.6. Absolute levels of acetylcholine release.

Absolute levels of ACh release in the ventral hippocampus accompanying inhibitory avoidance training in young and old rats. Baseline and training-associated increases in ACh release were significantly lower in old compared to young rats (Ps < 0.05 young vs. old). B=baseline samples; P=post-training samples.

CHAPTER 3: AGE-RELATED IMPAIRMENTS IN MEMORY AND IN CREB AND pCREB EXPRESSION IN HIPPOCAMPUS AND AMYGDALA FOLLOWING INHIBITORY AVOIDANCE TRAINING[2]

This experiment examined whether age-related changes in CREB and pCREB contribute to the rapid forgetting seen in aged animals. Young (3-month-old) and aged (24-month-old) Fischer-344 rats received inhibitory avoidance training with a low (0.2 mA, 0.4 s) or moderate (0.5 mA, 0.5 s) foot shock; memory was measured 7 days later. Other rats were euthanized 30 min after training, and CREB and pCREB expression levels were examined in the hippocampus, amygdala, and piriform cortex using immunohistochemistry. CREB levels decreased with age in the hippocampus and amygdala. After training with either shock level, young rats exhibited good memory and increases in pCREB levels in the hippocampus and amygdala. Aged rats exhibited good memory for the moderate but not the low shock but did not show increases in pCREB levels after either shock intensity. These results suggest that decreases in total CREB and in pCREB activation in the hippocampus and amygdala may contribute to rapid forgetting in aged rats. After moderate foot shock, the stable memory in old rats together with absence of CREB activation suggests either that CREB was phosphorylated in a spatiotemporal pattern other than analyzed here or that the stronger training conditions engaged alternate mechanisms that promote long-lasting memory.

[2] This chapter appeared in its entirety in the journal Mechanisms of Ageing and Development and is referred to later in this dissertation as Morris and Gold, 2012a. Morris, K.A., and Gold, P.E. (2012a). Age-related impairments in memory and in CREB and pCREB expression in hippocampus and amygdala following inhibitory avoidance training. Mech. Ageing Dev., 133, 291-299. This article is reprinted with the permission of the publisher.

3.1. INTRODUCTION

As seen in humans, rats and mice exhibit age-related impairments in learning and memory on many tasks. Often, the impairments are characterized in terms of rapid forgetting, in which aged rodents perform similarly to young adult rodents on memory tests soon after training, but have poor memory at later times as compared to young rodents (Barnes, 1991; Foster, 1999; Gold, 2001, 2005; Korol, 2002; Winocur, 1988). There are many examples of accelerated forgetting in aged rodents, with specific time courses that differ by task. In particular, memory for inhibitory avoidance training remains stable in young adult rats for weeks after training but decays within hours to days in old rats. Importantly, the age-related difference in forgetting rate is apparent despite similar or lower foot shock perceptual thresholds in old rats (Foster and Kumar, 2007; Frye et al., 2010; Gold et al., 1982; Morris et al., 2010). Rapid forgetting is also evident in a variety of other tasks, including the water maze (Burke et al., 2008; Gage et al., 1984; Mabry et al., 1996; Rapp et al., 1987), reward reduction (Salinas and Gold, 2005), social transmission of food preference (Countryman and Gold, 2007), visual discriminated avoidance (Gold et al., 1982), Barnes circular maze (Barnes and McNaughton, 1985), spatial reversal (Zornetzer et al., 1982), spontaneous alternation (Da Silva Costa-Aze et al., 2011; McNay and Gold, 2001; Stone et al., 1997), odor-reward association (Roman et al., 1996), and eye-blink classical conditioning (e.g. Solomon et al., 1995; Woodruff-Pak et al., 2007). The breadth of examples of rapid forgetting suggests that this is a key characteristic of age-related changes in memory.

Rapid forgetting seen during aging is analogous to similar findings seen after many treatments that interfere with cell and molecular processes associated with the

formation of new memories. Rapidly decaying memory is seen after administration of protein synthesis inhibitors, ERK/MAPK inhibitors, and inhibitors of transcription factors, such as cAMP response element binding protein (CREB), and is also seen in several knockout and transgenic mice with alterations aimed at these and other molecular targets (e.g. Alberini, 2009; Apergis-Schoute et al., 2005; Costa-Mattioli and Sonenberg, 2008; Goelet et al., 1986; Guzowski et al., 2000; Houpt and Berlin, 1999; Izquierdo et al., 2006; Kandel, 2001; McGaugh, 2000; Taubenfeld et al., 2001; Trifilieff et al., 2006). The parallels between the rapid forgetting in these experiments and those seen in aged rodents suggest that similar cellular mechanisms may be involved.

Of particular relevance to the experiments reported here, considerable evidence suggests that CREB is a transcription factor important to the formation of durable memories, perhaps converting rapidly decaying memories and short-term potentiation to more permanent forms (Benito and Barco, 2010; Carlezon et al., 2005; Colombo et al., 2003; Josselyn, 2010; Silva et al., 1998; Taubenfeld et al., 1999; Yin and Tully, 1996). For example, interfering with CREB function via mutations or inhibitors generally disrupts memory assessed at long but not short times after training (Bourtchuladze et al., 1994; Brightwell et al., 2005, 2008; Frankland et al., 2004; Guzowski and McGaugh, 1997; Josselyn et al., 2004; Yin et al., 1994), and these findings are analogous to the rapid forgetting seen in aged rodents. Several studies have examined CREB functions in aged rodents (cf. Lund et al., 2004). One consistent finding is that aged rodents are impaired in training-related activation of phosphorylated CREB (pCREB) in the hippocampus (Countryman and Gold, 2007; Kudo et al., 2005; Monti et al., 2005; Porte et al., 2008a; Xu et al., 2010). Several studies of chronically administered treatments

have identified substances that can attenuate age-related memory impairments while also enhancing CREB phosphorylation (Assunção et al., 2010; Li et al., 2009; Trofimiuk et al., 2010; Xu et al., 2010). Mouravlev et al. (2006) demonstrated that somatic gene transfer of CREB protein into the hippocampus of young adult rats prevented later formation of age-associated memory impairments. Also, Brightwell et al. (2004) found that aged rats with poor spatial memory have lower hippocampal CREB levels than do those with good spatial memory. However, prior aging studies have apparently not examined whether altering training conditions to promote stable memory formation in aged rodents would reverse deficits in CREB activation.

The present report tested the hypothesis that age-related differences in the expression and activation of CREB and pCREB may contribute to the rapid forgetting that is characteristic of aged rodents. Therefore, we used an inhibitory avoidance task in which the rate of forgetting is accelerated in aged rats. In addition, increases in the aversive component of training result in better maintenance of memory, providing a comparison of conditions in which memory is rapidly or slowly forgotten. Thus, in the experiments reported here, rats were trained with either a low intensity foot shock, which led to age-related rapid forgetting, or a moderate intensity foot shock, which led to stable memory formation in both young and old rats. In past studies of aging and memory, CREB and pCREB expression were assessed only in the hippocampus. In the present experiments, CREB and pCREB expression levels were assessed with immunohistochemistry in brain sections collected 30 min after training from the amygdala and piriform cortex, as well as from hippocampal dentate gyrus, area CA3, and area CA1 (see Fig. 3.1).

3.2. MATERIALS AND METHODS

3.2.1. Subjects

Young adult (3–4 mo.) and old (24–25 mo.) male Fischer-344 rats (Taconic Farms, Germantown, NY) were individually housed in translucent cages with a 12-h light/12-h dark cycle (lights on at 07:00 h) and *ad libitum* access to food and water. Animal pain and discomfort were minimized, and all experiments were conducted in accordance with animal care guidelines established by the National Institute of Health and the University of Illinois, which is fully accredited (AAALAC).

3.2.2. Inhibitory Avoidance Training

Rats were handled on 5 consecutive days for 5 min each day prior to behavioral training. All training and testing took place between 1200 and 1600 h. The inhibitory avoidance apparatus was a trough-shaped alleyway (91 cm long, 22.9 cm wide at the top, 7.6 cm wide at the bottom, and 15.2 cm deep) divided into lit (31 cm) and dark (60 cm) compartments by a sliding door that could be lowered through the floor. Each rat was placed in the lit chamber facing the door. When the rat turned completely around, the door was lowered to a height approximately 2 cm above the floor. When the rat again turned toward the door, a timer was started to record the latency to enter the dark chamber. Upon entering the dark chamber, the rat received a brief foot shock (either 0.2 mA, 0.4 s, or 0.5 mA, 0.5 s) and the door was closed to prevent re-entry into the lit chamber. The rat was then returned to the holding cage. For the initial behavioral characterization, retention latencies (max of 600 s) were tested 7 days after training using the same procedure. To measure CREB and pCREB levels, rats were euthanized

30 min after training and immunostaining procedures were used at a later time. Groups for the CREB and pCREB measures also included a cage control group, in which rats were left in their home cage until euthanasia, and a no shock group, in which rats were placed in the training apparatus and allowed to cross from the lit to dark compartment, but did not receive a foot shock. Ns = 4 for the initial behavioral characterization. For the immunohistochemistry studies, N = 7 for the young cage control group and Ns = 4 for all other groups.

3.2.3. Perfusion and Brain Slicing

Rats were deeply anesthetized with an overdose i.p. injection of sodium pentobarbital (Sigma–Aldrich, St. Louis, MO) and then perfused intracardially with 80 ml of 0.1 M phosphate-buffered saline followed by 80 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Rats were decapitated and the brains were removed and placed into 4% paraformaldehyde in 0.1 M PB for ~72 h. The brains were transferred to 20% glycerol in 0.1 M PBS for ~48 h. Frozen sections (40 mM) were collected at -30 ^{o}C with a Leica 1800 cryostat (Leica Microsystems, Wetzlar, Germany). Slices through the dorsal hippocampus were collected and stored in a cryopreservative solution (250 mM 40 KD polyvinylpyrrolidone, 880 mM sucrose, 30% v/v ethylene glycol, 50 mM sodium phosphate) at -20^{o} C.

3.2.4. Immunohistochemistry

All steps took place at room temperature. All reactions were performed in duplicate, using alternating slices for CREB and pCREB staining. Slices were washed three times for 10 min each time in 0.05 M PBS initially and between all subsequent

steps. Slices were first incubated in blocking solution (1% H_2O_2, 1% NGS, 0.02% triton x-100, 0.05 M PBS) for 10 min. They were transferred to a preincubation solution (2% NGS, 0.4% triton x-100, 0.05 M PBS) for 20 min and then incubated overnight in a solution (1% NGS, 0.4% triton x-100, 0.05 M PBS) containing a rabbit primary antibody for CREB or Ser-133 phosphorylated CREB (Millipore, Billerica, MA) diluted 1:4000. The next day, the slices were placed for 1 h in a solution (1% NGS, 0.2% triton x-100, 0.05 M PBS) containing a goat anti-rabbit biotinylated secondary antibody (Santa Cruz, Santa Cruz, CA). They were next incubated for 30 min with ABC reagent (Vector, Burlingame, CA) in 0.05 M PBS, followed by incubation with DAB substrate (Vector) for 4 min. Slices were mounted onto slides and allowed to dry overnight. The next morning, slices were dehydrated with a graded ethanol series of washes, then coverslipped using DPX mountant (Sigma–Aldrich).

3.2.5. Image Acquisition and Analysis

Sections were imaged using a Leica DM 6000B/CTR6000 light microscope and a Leica DFC350 FX video camera, which was interfaced to a PC computer. This system was used in conjunction with Image-Pro software (Media Cybernetics, Inc., Bethesda, MD) for image acquisition and for correction of unevenness in illumination across images. Image J software (NIH, Bethesda, MA) was used to quantify the optical density of CREB and pCREB staining in subregions of the hippocampus, amygdala, and piriform cortex. Fig. 3.1 shows the specific regions that were analyzed. A statistical thresholding method in Image J was used to ensure that only specifically labeled cells were being measured. For each image, the optical density of a nearby region with no or little specific staining was calculated and used for background subtraction.

3.2.6. Statistical Analyses

All analyses were performed using Statview software. The optical densities of CREB and pCREB immunostaining were analyzed using two-way ANOVAs with post hoc Fisher PLSD tests where appropriate. Behavioral results were analyzed using a non-parametric Kruskal-Wallis one-way analysis of variance, followed by Mann-Whitney tests for individual comparisons.

3.3. RESULTS

3.3.1. Behavioral Performance on the Inhibitory Avoidance Task.

Fig. 3.2 shows training latencies (Left) and 7-day retention latencies (Right) of young and old rats trained on an inhibitory avoidance task. There were no significant age-related differences in training latencies. Young rats had maximum median retention latencies of 600 s following a 0.2 mA, 0.4 s or 0.5 mA, 0.5 s foot shock. The old rats had a maximum median retention latency following the moderate intensity foot shock, but a median retention latency of only 66.9 s with the lower intensity foot shock. Non-parametric analysis of variance revealed a significant group effect ($H = 8.7$, $p < .05$). Post hoc analyses showed that old rats receiving the low intensity foot shock had significantly lower retention latencies compared to each of the other three groups ($ps < .05$).

3.3.2. CREB and pCREB Immunostaining Following Training.

Differences in immunostaining of pCREB, total CREB, as well as pCREB:CREB ratios, were examined in young and old rats 30 min after inhibitory avoidance training with a low (0.2 mA, 0.4 s) or moderate (0.5 mA, 0.5 s) foot shock. These groups were

compared to cage controls and to no shock controls, which were trained on inhibitory avoidance without the foot shock. These results are summarized in Table 3.1.

3.3.2.1. Hippocampus: Dentate Gyrus

Fig. 3.3 (A and B) shows pCREB immunostaining in the dentate gyrus region of the hippocampus. Activation of CREB by training was evident in young but not aged rats. A two-way ANOVA revealed a main effect of training on pCREB staining ($F_{(3,27)}$ = 7.30, $p < .01$). In young rats, training with a 0.2 mA foot shock significantly enhanced pCREB staining compared to cage controls ($p < .01$). Training with a 0.5 mA foot shock enhanced pCREB staining compared to cage control, no shock, and 0.2 mA groups (ps < .03). In old rats, there were no significant increases in pCREB staining in response to training at either foot shock intensity. There was a main effect of age on pCREB ($F_{(1,27)}$ = 18.17, $p < .001$), indicating lower pCREB staining in old compared to young rats. Post hoc tests were used to identify age-related differences within training groups (e.g. young cage controls vs. old cage controls). There were significant age-related differences between the young and old rats in the 0.2 mA and 0.5 mA shock groups (ps < .05). In addition to the training and age effects, there was a significant age by training interaction ($F_{(3,27)} = 3.58$, $p < .05$).

There was an overall main effect of age ($F_{(1,27)} = 4.36$, $p < .05$) on CREB immunostaining, with lower staining observed in old rats (Fig. 3.3C). There were no main effects of training on CREB staining ($F_{(3,27)} = 0.24$).

There was a main effect of training ($F_{(3,27)} = 4.76$, $p < .01$) but not age ($F_{(1,27)}$ = 1.38) on pCREB:CREB ratios (Fig. 3.3D). In young rats, training with a 0.2 mA shock increased pCREB:CREB ratios above those of cage control rats ($p < .03$). Training with

a 0.5 mA shock increased pCREB:CREB ratios compared to both cage control and no shock groups (ps < .03). In old rats, training did not significantly alter pCREB:CREB ratios, regardless of the foot shock intensity.

3.3.2.2. Hippocampus: Area CA3

There was a main effect of age ($F_{(1,27)} = 6.45$, $p < .03$) but not training ($F_{(3,27)} = 2.40$) on pCREB immunostaining in area CA3 (Fig. 3.4A and B). However, post hoc analyses revealed no significant differences between groups. There was also a main effect of age ($F_{(1,27)} = 22.74$, $p < .0001$) but not training ($F_{(3,27)} = 0.06$) on CREB staining (Fig. 3.4C). Post hoc analyses revealed significantly lower CREB staining in old compared to young rats in the cage control, 0.2 mA shock, and 0.5 mA shock groups (ps < .05), but not in the no shock group. There was a main effect of age ($F_{(1,27)} = 4.87$, $p < .05$) on pCREB:CREB ratios (Fig. 3.4D), suggesting higher ratios in old rats. There were no training effects on pCREB:CREB ratios ($F_{(3,27)} = 0.78$).

3.3.2.3. Hippocampus: Area CA1

In area CA1, the effects on pCREB, CREB, and pCREB:CREB ratios were very similar to those seen in the dentate gyrus, and are therefore not presented in detail here.

3.3.2.4. Basolateral Amygdala

Both training ($F_{(3,27)} = 5.19$, $p < .01$) and age ($F_{(1,27)} = 36.89$, $p < .0001$) significantly affected pCREB immunostaining in the basolateral amygdala (Fig. 3.5A and B). In young rats, training with a 0.2 mA foot shock increased pCREB staining compared to cage controls ($p < .05$). Training with a 0.5 mA foot shock increased

pCREB staining compared to both cage and no shock controls (ps < .01). In old rats, training did not significantly alter pCREB staining, regardless of the shock intensity. Compared to cage controls, age-related deficits in pCREB activation were evident in the no shock, 0.2 mA shock, and 0.5 mA shock groups (ps < .05). There was also a significant age by training interaction ($F_{(3,27)} = 3.29$, $p < .05$).

There was a main effect of age ($F_{(1,27)} = 21.39$, $p < .0001$) but not training ($F_{(3,27)} = 0.20$) on CREB immunostaining (Fig. 3.5C). Post hoc analyses revealed significantly lower CREB staining in old rats in the cage control and 0.5 mA shock groups (ps < .05).

There was a main effect of training ($F_{(3,27)} = 3.39$, $p < .05$) but not age ($F_{(1,27)} = 2.36$) on pCREB:CREB ratios (Fig. 3.5D). In young rats, the 0.2 mA shock group had significantly higher ratios compared to cage control rats ($p < .05$). The 0.5 mA shock group had significantly higher ratios compared to both the cage control and no shock groups (ps < .05). In old rats, training did not have a significant effect on pCREB:CREB ratios.

3.3.2.5. Lateral Amygdala

In the lateral amygdala, the effects on pCREB, CREB, and pCREB:CREB ratios were similar those seen in the basolateral amygdala, with the major exception being lack of a training effect on pCREB:CREB ratios in the lateral amygdala (see Table 3.1). Therefore, these results are not presented in detail here.

3.3.2.6. Piriform cortex

There were no main effects of age ($Fs_{(1,27)} < 3.67$) or training ($Fs_{(3,27)} < 0.07$) on pCREB, CREB, or pCREB:CREB ratios in the piriform cortex (Fig. 3.6).

3.4. DISCUSSION

3.4.1. Age-related Differences in Memory

Training with a low intensity foot shock produced stable 7-day memory in young rats, but led to poor 7-day memory in old rats. These results support prior work demonstrating rapid age-related forgetting following inhibitory avoidance training (Gold et al., 1982; Morris et al., 2010). In particular, Gold et al. (1982) trained rats with a low intensity foot shock and training-testing intervals ranging from 2 h to 6 weeks. Young rats had stable memory for at least 3 weeks after training. Old rats had comparable or even better memory than young rats 2 h after training. However, the old rats exhibited slight deficits after 1 day, and were significantly impaired after 7 days. Thus, old rats exhibited rapid forgetting for inhibitory avoidance, with memory intact for hours after training, but with memory impairments emerging during the days after training. Importantly, this operational definition of forgetting may reflect memories with variable time courses depending on the salience of the initiating events.

In contrast to the results with the low foot shock, training with a moderate intensity foot shock resulted in stable 7-day memory in both young and old rats. These results are similar to findings by Gold et al. (1982), and indicate that forgetting rates in old rats can be slowed by increasing the saliency of the training foot shock, perhaps with concomitant increases in neural modulators of memory associated with the higher training-related arousal levels. Together, the behavioral results presented here provide a good model with which to examine changes in CREB expression and activation that might contribute to rapid forgetting in aged rats.

3.4.2. Age-Related Differences in CREB and pCREB Expression at Baseline

At baseline, CREB levels were lower in aged vs. young adult rats in area CA3 and in the basolateral and lateral amygdala. Comparable changes were not evident in the dentate gyrus, area CA1, or piriform cortex, although the latter brain region had high CREB expression levels as seen previously (Ferrer et al., 1996; Herdegen et al., 1993). The age-related reductions in hippocampal CREB observed here are consistent with past findings revealing decreased expression of hippocampal CREB in old Fischer-344 rats (Countryman and Gold, 2007). Other studies have shown age-related reductions in CREB levels in whole hippocampal homogenates from Long Evans (Brightwell et al., 2004) and Wistar rats (Trofimiuk et al., 2010). The age-related decreases in CREB expression in the amygdala have not, to our knowledge, been noted before. The decreased expression of CREB in the hippocampus and amygdala with age, as well as the decreased training-related activation of CREB described below, may contribute to age-related memory impairments. The memory impairments are analogous to those seen in CREB knockout mice and in rats and mice treated with CREB antisense oligonucleotides, RNA interference, or dominant negative mutations, any of which impairs the formation of long-lasting memory and reduces the durability of long-term potentiation (Bourtchuladze et al., 1994; Canal et al., 2008; Florian et al., 2006; Guzowski and McGaugh, 1997; Josselyn, 2010; Peters et al., 2009; Pittenger et al., 2002; Won and Silva, 2008).

Importantly, age-related changes in CREB, as well as changes in expression of pCREB described below, do not likely reflect differences in cell numbers or cell morphology between young and old rats. Studies utilizing stereological methods for

quantification have found that neuron numbers are preserved in the hippocampus of old rats (Gallagher et al., 2003; Rapp and Gallagher, 1996; Rasmussen et al., 1996). Likewise, another study reported no significant volume or cell loss in the hippocampus or amygdala of aged mice (Von Bohlen und Halbach and Unsicker, 2002).

The age-related decreases in CREB levels in several brain regions suggest that some estimates of age-related changes in behaviorally elicited increases in pCREB levels may reflect the loss of CREB per se. Several recent studies of changes during aging in pCREB levels in the hippocampus have measured only pCREB or have used total CREB levels to normalize for changes in pCREB (e.g. Assunção et al., 2010; Hattiangady et al., 2005; Li et al., 2009; Trofimiuk et al., 2010; Xing et al., 2010; Xu et al., 2010; Zhao et al., 2009). The present results suggest that quantifying total CREB levels is important to interpret correctly age- or activity-dependent changes in pCREB in old rats. Further, using CREB levels for normalization in aging studies may make it difficult to compare directly results across age groups, since total CREB levels may decline with age in a brain area-specific manner.

3.4.3. Age-Related Differences in pCREB Expression in Response to Training

CREB phosphorylation in the piriform cortex has been shown to be responsive to several stimuli (Alvarez-López et al., 2004; Dere et al., 2008; Estrada and Isokawa, 2009; Kim et al., 2006; Pandey et al., 2001a,b; Wang et al., 2007). However, this is the first study, to our knowledge, to examine CREB activation in the piriform cortex after inhibitory avoidance training, revealing no evidence for pCREB increases after training in either young or aged rats. The results contrast with those showing c-Fos expression

and ERK activation in piriform cortex induced by active avoidance training in young adult rats (Radwanska et al., 2010).

A striking result was that there were substantial age-related differences in activation of CREB in response to training in the hippocampus and amygdala. In young adult rats, pCREB expression in the dentate gyrus, area CA1, and the lateral and basolateral amygdala increased 30 min after training with a low foot shock as compared to expression levels in young cage controls. Training with a moderate intensity foot shock further enhanced pCREB levels in the same brain regions. In parallel groups of young rats, both shock intensities produced high avoidance latencies in memory tests 7 days after training. These results are consistent with the view that, in young adult rats, CREB activation may be an important component or marker of long-term memory processes occurring after training. The results obtained in hippocampal regions agree with a number of other studies showing increased hippocampal CREB phosphorylation following inhibitory avoidance training in young adult rats, which can be abolished by treatments that interfere with memory processes (Cammarota et al., 2000; Taubenfeld et al., 1999, 2001; Viola et al., 2000). The amygdala results also agree with several studies demonstrating enhanced CREB phosphorylation in the basolateral and/or lateral amygdala following fearful or stressful stimuli (Bilang-Bleuel et al., 2002; Hubbard et al., 2007; Ilin and Richter-Levin, 2009; Kogan and Richter-Levin, 2008; Lin et al., 2003; Saha and Datta, 2005; Shen et al., 2004; Stanciu et al., 2001).

In marked contrast to the results obtained with young adult rats, aged rats did not show significant increases in pCREB expression in any brain region after training with either the low or moderate shock intensity. In old rats, pCREB levels in the dentate

gyrus, area CA1, and lateral and basolateral amygdala, i.e. those areas responsive to training in young rats, were similar to those of aged cage controls and were generally significantly lower than those in corresponding groups and samples of young rats. Viewed across all brain areas, age-related decreases in total CREB cannot entirely account for these impairments in CREB phosphorylation, since basal CREB levels in area CA1 and the dentate gyrus were similar between young and old rats. Rather, the findings suggest that age-related impairments upstream to CREB activation may be an important contributor to age-related impairments in memory. This interpretation is consistent with recent evidence that overexpression of CREB in the hippocampus improves memory in young but not middle-aged rats (East et al., 2011), perhaps due to an inability of middle aged rats to engage upstream activators of CREB phosphorylation when needed.

The present results are the first to identify substantial age-related impairments in CREB activation in the amygdala and hippocampus after inhibitory avoidance training. The failure to activate CREB in the amygdala may have significant consequences to modulation of memory in other neural systems, such as the hippocampus, and may contribute to the decreased pCREB after training in the hippocampus. Consistent with this possibility, intra-amygdala injections of beta-adrenergic agonists and antagonists, drugs that enhance and impair memory respectively, increase and decrease expression of activity-regulated cytoskeletal protein (Arc) in the hippocampus in conjunction with modulation of memory (McIntyre et al., 2005). These findings suggest that age-related deficiencies in amygdala modulation of hippocampal functions may be important in mediating the rapid forgetting and rapid decay of neural plasticity seen in aged rodents.

The present results also agree with a number of studies showing age-related impairments in CREB activation in a variety of hippocampal-dependent tasks (Countryman and Gold, 2007; Kudo et al., 2005; Monti et al., 2005; Porte et al., 2008a; Xu et al., 2010). Recent studies also indicate that long-term administration of certain compounds, including procyandins (Xu et al., 2010), green tea (Assunção et al., 2010; Li et al., 2009), and St. John's wort (Trofimiuk et al., 2010), can reverse age-related deficits in CREB activation in concert with memory enhancement. Thus, these findings are consistent with the view that a failure to activate CREB after training contributes to age-related memory impairments.

The present experiments included a condition, moderate intensity foot shock used during training, which tested further the importance of CREB activation for long-lasting memory. On the basis of past findings (Gold et al., 1982), we expected the moderate shock condition to result in longer maintenance of memory for inhibitory avoidance training, a result confirmed here. We further expected that the moderate training shock would also result in greater CREB activation that would parallel the better memory scores. This result was clearly not evident: Raising the foot shock intensity reversed age-related impairments in 7-day memory but did not increase CREB phosphorylation in the hippocampus or amygdala of old rats, as it did in young rats. Thus, CREB activation was dissociated from memory formation in old rats trained with the moderate foot shock intensity.

There are several possible explanations for the apparent dissociation between CREB activation and memory formation. In the present experiment, measurements of CREB activation were limited to a single time point, 30 min after training. Although some

studies suggest there is rapid and long-lasting CREB activation following training (e.g. Taubenfeld et al., 2001, 1999), others have observed specific and even delayed time windows of CREB phosphorylation after training (Bernabeu et al., 1997; O'Connell et al., 2000). Therefore, an earlier or later time point might reveal training-related increases in pCREB levels that would be associated with the stable memory observed here after training with the moderate shock intensity. It is also possible that the quantitative histochemistry methods used here were not sufficiently sensitive to reveal increases after the moderate shock intensity. Given the greater increase in pCREB expression levels seen in young rats after the moderate shock intensity, this seems unlikely, though the possibility cannot be fully discounted. Although pCREB activation in young rats appeared more or less uniform across large numbers of cells, such widespread activation may not be necessary for memory formation. For instance, Han et al. (2007, 2009) found that altering CREB function in small numbers of sparsely distributed cells in the lateral amygdala can affect formation of auditory fear memory (Han et al., 2007, 2009; Zhou et al., 2009). Sparse distribution of critical neurons expressing CREB activation would likely be missed using the immunohistochemistry methods employed here. Utilization of a cell counting strategy or of more quantitative techniques, such as ELISAs or western blots, may be helpful in providing corroborative molecular evidence of the optical density measurements presented here.

Another possibility is that the dissociation between CREB and memory may indicate that CREB activation is not required for memory formation in old rats. This view is consistent with a number of studies showing stable formation of memory and long-term potentiation in CREB knockout and mutant mice (Balschun et al., 2003; Gass et

al., 1998; Rammes et al., 2000). Although several studies do indicate deficits in long-term memory in CREB mutant mice (Bourtchuladze et al., 1994; Sekeres et al., 2010), these deficits can often be reversed by altering training procedures or by administering memory-enhancing agents (Frankland et al., 2004; Kogan et al., 1997; Canal et al., 2008). Further, the idea that CREB activation is not necessary for the formation of stable memories after the moderate shock is consistent with the possibility that the moderate shock level recruits additional molecular components of memory formation compared to those seen with the lower shock. In young rats, the behavioral measure of memory is maximal at both shock intensities, thereby obscuring the utility of additional molecular mechanisms. However, in aged rats, the stable memory after training with the moderate shock, coupled to the absence of CREB activation, may reflect the importance of these additional molecular mechanisms. In this regard, the study of stable memory in aged rats may be a condition favorable to investigation of these additional factors.

3.5. FIGURES

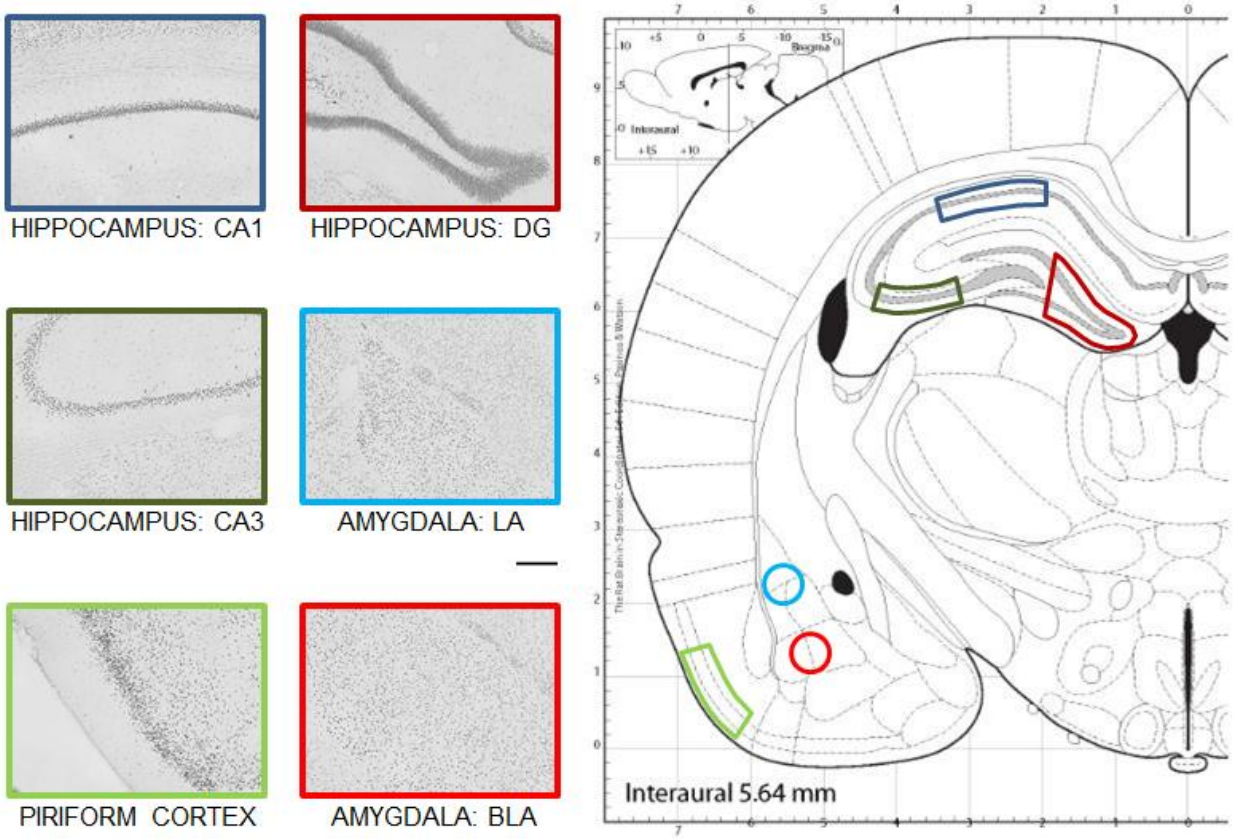

Figure 3.1. Illustration of the areas targeted in this study.

The targeted areas were the dentate gyrus (DG), CA3, and CA1 of the hippocampus, the lateral (LA) and basolateral (BLA) nuclei of the amygdala, and the piriform cortex. Photomicrographs show representative CREB immunoreactivity in each region. Scale bar = 200 mm. Adapted with permission from Paxinos and Watson (2005).

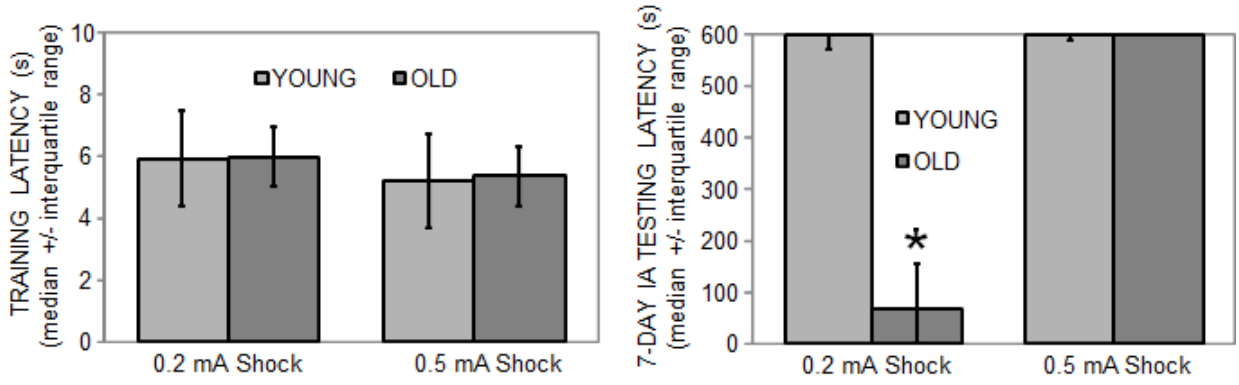

Figure 3.2. Training and 7-day retention latencies in young and old rats trained on inhibitory avoidance with a 0.2 or 0.5 mA foot shock.

(Left) There were no significant differences in training latencies across groups. (Right) Old rats had significantly lower 7-day retention latencies following the low intensity foot shock than did all other groups (*) ps < .05. Note that old rats exhibited maximal retention latencies after training with the higher foot shock intensity.

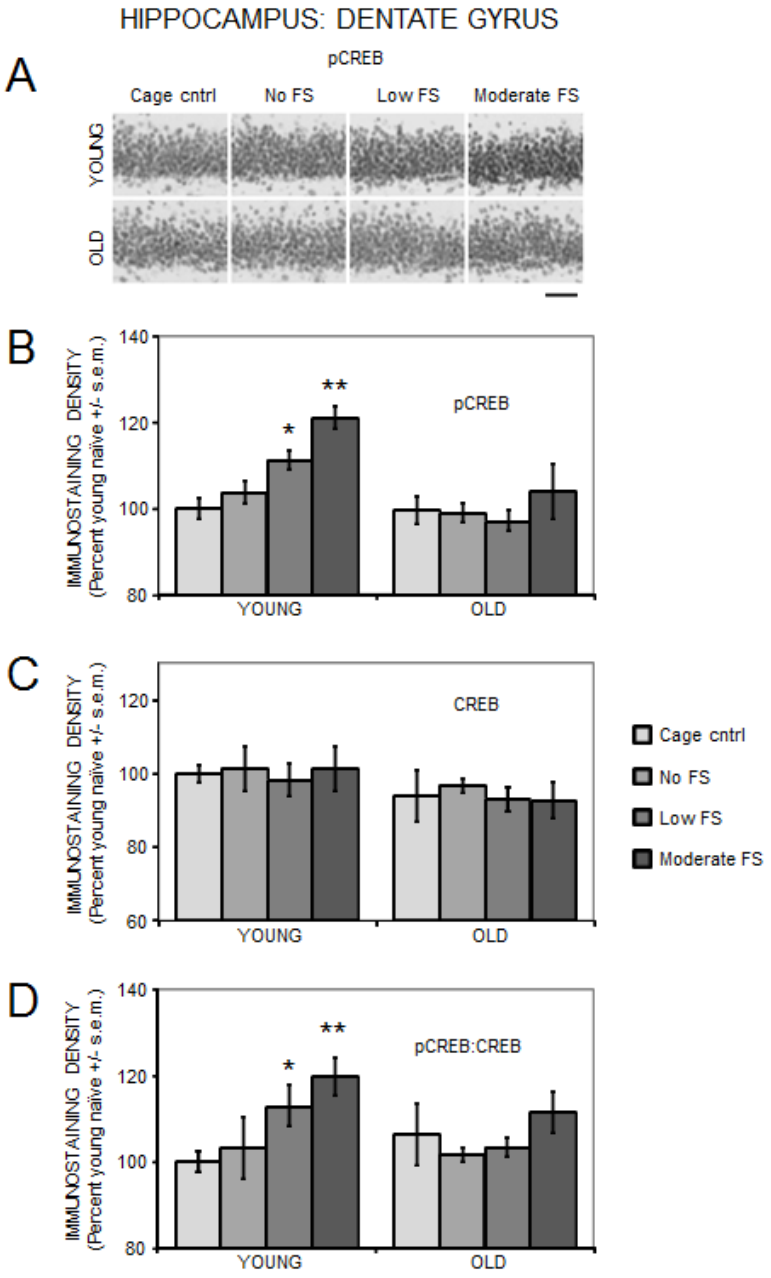

Figure 3.3. Age- and training-associated differences in pCREB and CREB immunoreactivity in the dentate gyrus of the hippocampus.

(A) Representative photomicrographs of pCREB immunostaining. Scale bar = 50 mm. (B) Young rats had significantly enhanced pCREB levels following training with a low or moderate intensity foot shock, whereas old rats had training-related deficits in pCREB activation. (*) $p < .01$ vs. young cage controls. (**) $ps < .05$ vs. young cage control, no shock, and 0.2 mA. (C) Total CREB levels were similar between young and old rats and not altered by training. (D) Young rats had significantly higher ratios of pCREB:CREB after training with a low or moderate intensity foot shock, whereas old rats had training-related deficits in pCREB:CREB ratios. (*) $p < .05$ vs. young cage controls. (**) $ps < .05$ vs. young cage controls and young no shock.

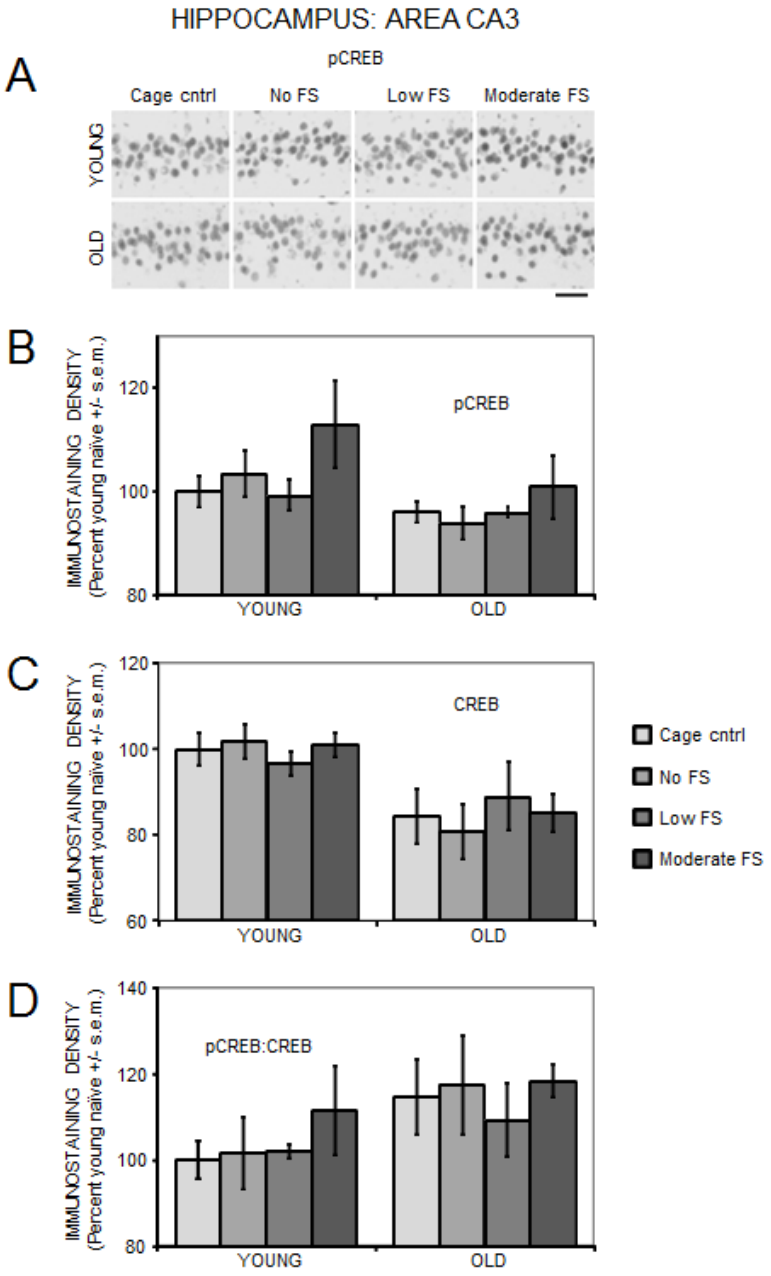

Figure 3.4. Age- and training-associated differences in pCREB and CREB immunoreactivity in area CA3 of the hippocampus.

(A) Representative photomicrographs of pCREB immunostaining. Scale bar = 50 mm. (B) pCREB levels were similar between young and old rats and not significantly altered by training. (C) There were significantly lower CREB levels in old compared to young rats. (D) There were no significant age- or training-related differences in pCREB:CREB ratios.

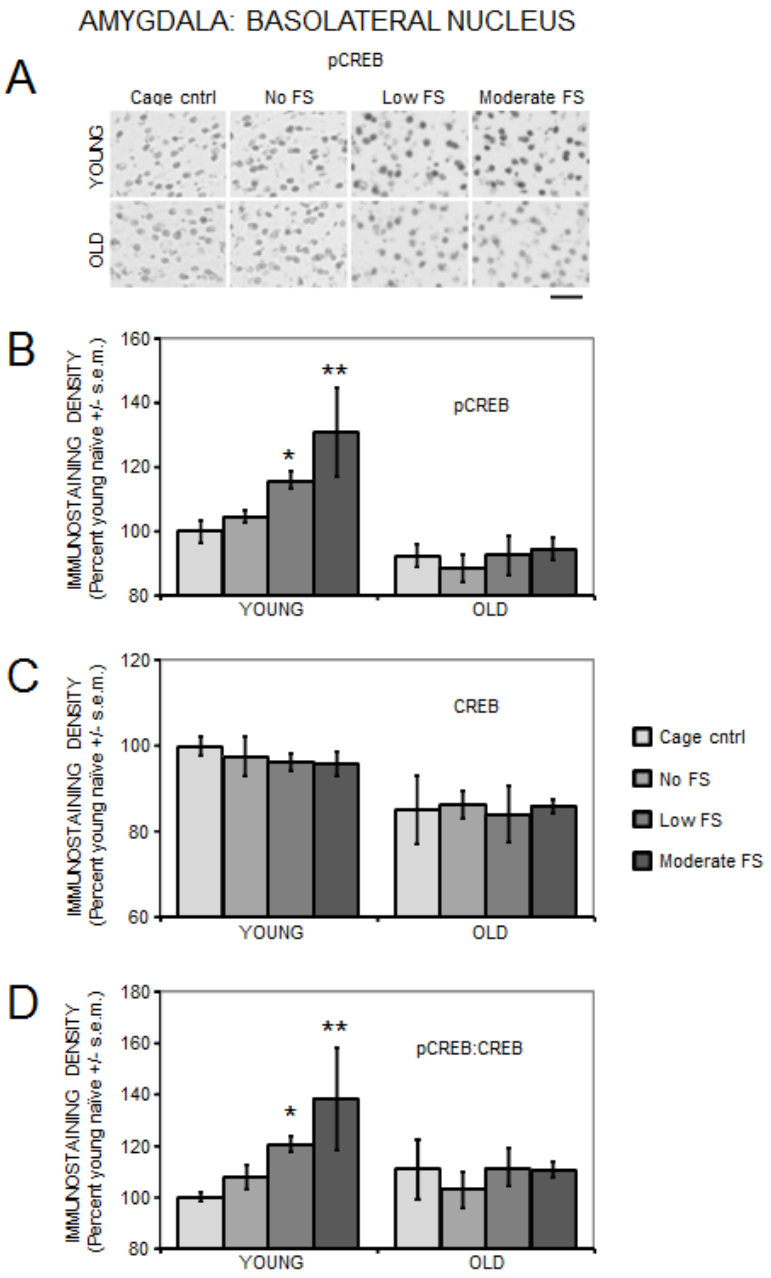

Figure 3.5. Age- and training-associated differences in pCREB and CREB immunoreactivity in the basolateral amygdala.

(A) Representative photomicrographs of pCREB immunostaining. Scale bar = 50 mm. (B) Young rats had significantly enhanced pCREB levels following training with a low or moderate intensity foot shock, whereas old rats had training-related deficits in pCREB activation. (*) $p < .05$ vs. young cage controls. (**) $ps < .01$ vs. young cage controls and no shock. (C) Total CREB levels were significantly lower in old compared to young rats. (D) Young rats had significantly higher ratios of pCREB:CREB after training with a low or moderate intensity foot shock, whereas old rats had training-related deficits in pCREB:CREB ratios. (*) $p < .05$ vs. young cage controls. (**) $ps < .05$ vs. young cage controls and no shock.

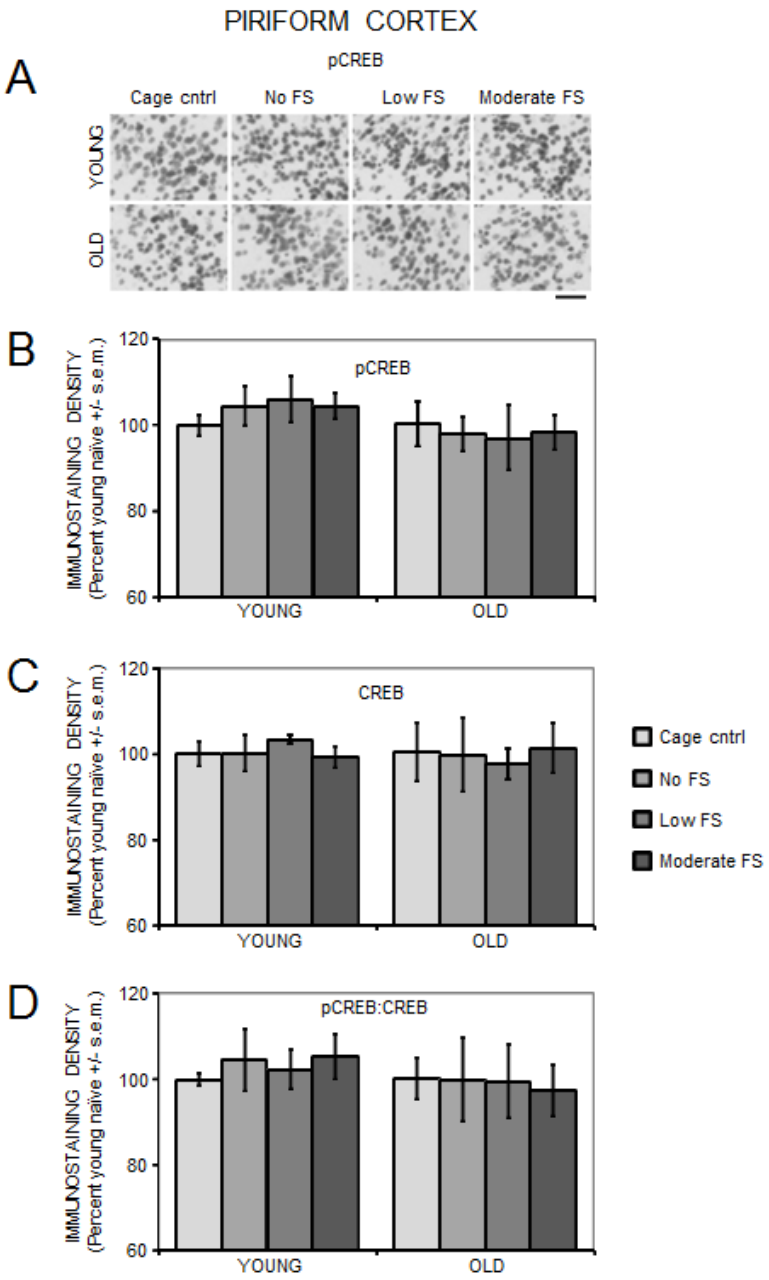

Figure 3.6. Age- and training-associated differences in pCREB and CREB immunoreactivity in the piriform cortex.

(A) Representative photomicrographs of pCREB immunostaining. Scale bar = 50 mm. There were no age- or training-related differences in pCREB (B), CREB (C), or ratio of pCREB:CREB (D).

Table 3.1. Summary of Immunohistochemistry Results.

	TRAINING EFFECT		AGE EFFECT
	YOUNG RATS	OLD RATS	
pCREB			
DG	✓		✓
CA1	✓		✓
CA3			
BLA	✓		✓
LA	✓		✓
PIR			
CREB			
DG			
CA1			
CA3			✓
BLA			✓
LA			✓
PIR			
pCREB:CREB			
DG	✓		
CA1	✓		
CA3			
BLA	✓		
LA			
PIR			

✓denotes significant main effect of age or training with foot shock, along with significant effect in at least one post hoc planned comparison; Brain regions analyzed were dentate gyrus (DG), area CA1, and area CA3 of the hippocampus, basolateral (BLA) and lateral (LA) amygdala, and piriform cortex (PIR).

CHAPTER 4: EPINEPHRINE AND GLUCOSE MODULATE TRAINING-RELATED CREB PHOSPHORYLATION IN OLD RATS: RELATIONSHIPS TO AGE-RELATED MEMORY IMPAIRMENTS

Epinephrine enhances memory in young adult rats, in part, by increasing blood glucose levels needed to modulate memory. In old rats, epinephrine is deficient at raising blood glucose levels and thus is only moderately effective at enhancing memory. The diminished function of this neuroendocrine response in old rats may alter downstream neurochemical and molecular mechanisms needed to upregulate memory processes. In the first experiment reported here, young adult and old rats were trained on an inhibitory avoidance task with immediate post-training injections of aCSF or glucose into the dorsal hippocampus. Old rats had significant memory impairments compared to young rats 7 days after training. Intrahippocampal injections of glucose reversed age-related deficits, improving memory scores in old rats to values seen in young rats. The second experiment examined age-related changes in training-related activation of the transcription factor CREB, which is widely implicated in memory formation and may act downstream of hormonal and metabolic signals. Young adult and old rats were trained on inhibitory avoidance with immediate post-training injections of saline, epinephrine, or glucose. After training, old rats had significant impairments in CREB phosphorylation in area CA1 and the dentate gyrus region of the hippocampus, and in the basolateral and lateral amygdala. Epinephrine and glucose attenuated age-related deficits in CREB phosphorylation, but were more effective in the amygdala and hippocampus, respectively. Together, these results support the view that age-related

changes in blood glucose responses to epinephrine contribute to memory impairments, which may be related to alterations in regional patterns of CREB phosphorylation.

4.1. INTRODUCTION

Memory impairments accompany healthy aging in a variety of species. The impairments often take the form of rapid forgetting of recently learned information in both humans (Gagnon and Belleville, 2011; Huppert and Kopelman, 1989; Munro Cullum et al., 1990; Park et al., 1988) and rodents (Barnes, 1991; Countryman and Gold, 2007; Foster, 1999; Gold, 2005, 2008; Gold et al., 1982; Korol, 2002; Quartermain et al., 1988; Salinas and Gold, 2005; Winocur, 1988; Zornetzer et al., 1982). Previous work in rodents suggests that rapid forgetting may reflect deficits in neurobiological mechanisms of memory formation initiated during or soon after training. For example, old rodents have training-related alterations in neurotransmitter release, calcium signaling, and gene expression within the brain, all of which may be interrelated and contribute to memory impairments (Burke and Barnes, 2006; Dickstein et al., 2007; Kelly et al., 2006; Mora et al., 2007; Toescu and Verkhratsky, 2007; Welsh and Gold, 1984; Yankner et al., 2008).

Changes outside of the brain may also make important contributions to age-related impairments in memory processes. Substantial evidence suggests that alterations in peripheral hormones and neuroendocrine systems during aging can alter central neurobiological processes, producing deficits in memory and synaptic plasticity (Conrad and Bimonte-Nelson, 2010; Foy, 2011; Frick, 2009; Janowsky, 2006; Korol and Gold, 2007; Lupien et al., 2009). In particular, numerous studies indicate that deficits in rising blood glucose levels after peripheral epinephrine release contribute to age-related

impairments in memory processes (Gold, 2005; Gold and Korol, 2010; Korol, 2002; Korol and Gold, 1998, 2007; Mabry et al., 1996; Messier, 2004). Epinephrine is a hormone released from the adrenal medulla as a response to arousal. In young adult rats, endogenous release of epinephrine facilitates stable memory formation for temporally associated events. Likewise, peripheral injections of epinephrine can convert short-lasting memories and the early phase of long-term potentiation (LTP) to more lasting forms (Korol and Gold, 2008). Since peripheral epinephrine cannot readily enter the brain from blood, epinephrine mediates its effects on memory largely by liberating glucose from liver glycogen stores, thus increasing the supply of glucose to the brain during cognitively demanding times (Gold, 2005, 2008; Gold and Korol, 2010; Korol and Gold, 2007). The additional provision of glucose to the brain provides energy to support memory processes, possibly by contributing to lactate production in astrocytes with provision to neurons during cognitive processing (Newman et al., 2011; Suzuki et al., 2011).

Several studies show that epinephrine is deficient at raising blood glucose levels in old rats. Though old rats exhibit higher circulating epinephrine levels following training or exposure to a novel environment, they do not show the parallel increases in blood glucose levels seen in young rats (Mabry et al., 1995a,b,c). Likewise, peripheral injections of epinephrine fail to increase blood glucose levels in old rats to the levels seen in young rats (Morris et al., 2010). These age-related deficits in blood glucose responses to epinephrine can affect downstream neurochemical and behavioral memory processes. One mechanism by which glucose may enhance memory is by increasing behaviorally-elicited release of the neurotransmitter acetylcholine (Gold,

2003; Kopf et al., 2001; Ragozzino and Gold, 1995; Ragozzino et al., 1996, 1998). In young adult rats, post-training injections of epinephrine or glucose enhance hippocampal acetylcholine release similarly while improving later memory for an inhibitory avoidance task. In contrast, glucose is more effective than epinephrine at enhancing acetylcholine release and improving memory in old rats (Morris et al., 2010). These results suggest that glucose administration in old rats may bypass the need for epinephrine to increase blood glucose levels and activate the downstream release of acetylcholine.

Age-related impairments in blood glucose responses to epinephrine may also affect downstream molecular processes, particularly those activated by cholinergic signaling. Several studies suggest that acetylcholine modulates phosphorylation of cAMP response element-binding protein (CREB) in the brain, most likely through activation of nicotinic acetylcholine receptors (Chang and Berg, 2001; Dajas-Bailador and Wonnacott, 2004; Hu et al., 2002). CREB is a transcription factor widely implicated in activity-dependent neuronal plasticity and the formation of durable memories (Alberini, 2009; Benito and Barco, 2010; Carlezon et al., 2005; Colombo et al., 2003; Silva et al., 1998; Taubenfeld et al., 1999; Yin and Tully, 1996). In young adult rats, interfering with CREB function via mutations or inhibitors generally disrupts long-term memory while leaving short-term processes intact (Bourtchuladze et al., 1994; Brightwell et al., 2005, 2008; Guzowski and McGaugh, 1997; Josselyn et al., 2004), mimicking age-related rapid forgetting. In old rats, there are significant age-related deficits in CREB activation after training in a variety of behavioral tasks, and these deficits correlate with memory impairments and rapid forgetting (Countryman and Gold,

2007; Kudo et al., 2005; Monti et al., 2005; Porte et al., 2008a; Xu et al., 2010). Several recent studies have shown that long-term exercise programs or chronic administration of various exogenous compounds can attenuate age-related impairments in CREB activation while improving performance in behavioral tasks (Aguiar et al., 2011; Assunção et al., 2010; Li et al., 2009; Trofimiuk et al., 2010; Xu et al., 2010). However, it is difficult to tell if these effects are specific to memory processes, given that the treatments were administered for several weeks or months prior to training. Few studies have examined how post-training administration of endogenous memory enhancing agents, such as epinephrine and glucose, may modulate memory and CREB function in old rats.

Of particular relevance to the current experiments, a recent study found that old rats are significantly impaired in CREB phosphorylation following inhibitory avoidance training, even when the foot shock intensity is raised to promote stable memory formation (Morris and Gold, 2012a). Similarly, other work has shown that old rats do not exhibit a foot shock-related rise in blood glucose levels, even with relatively high foot shock intensities (Mabry et al., 1995c). Together, these results suggest that old rats are significantly impaired in their ability to initiate CREB-mediated transcriptional processes, which may reflect deficits in blood glucose rises and associated impairments in acetylcholine release soon after training.

The present experiments utilized post-training designs to examine how deficits in blood glucose responses to epinephrine in old rats may contribute to impairments in memory and CREB activation. The first experiment tested the hypothesis that direct brain injections of glucose after inhibitory avoidance training could reverse age-related

memory impairments. The second experiment examined the hypothesis that glucose would be more effective than epinephrine at modulating downstream CREB phosphorylation in the brain, similar to glucose's greater efficacy in previously tested neurochemical and behavioral measures.

4.2. METHODS

4.2.1. Subjects

Young adult (3 to 4 mo.) and old (24 to 25 mo.) male Fischer-344 rats (Taconic Farms, Germantown, NY) were individually housed in translucent cages with a 12-h light/dark cycle (lights on at 07:00 h) and *ad libitum* access to food and water. Animal pain and discomfort were minimized. All experiments were conducted at the University of Illinois, which is fully accredited (AAALAC), in accordance with animal care guidelines established by the National Institute of Health.

4.2.2. Surgery

Rats were anesthetized with isoflurane and placed in a stereotaxic apparatus. Stainless steel guide cannulae (Plastics One, Roanoke, VA) were implanted bilaterally into the dorsal hippocampus of young [coordinates: - 3.2 mm from bregma; ± 3 mm lateral; - 1.9 mm deep from dura] and old rats [coordinates: - 3.4 mm from bregma; ± 3.1 mm lateral; - 2.1 mm deep from dura], according to the atlas of Paxinos and Watson (2005). Rats were monitored and allowed to recover for 7 days after surgery before further experimentation.

4.2.3. Inhibitory Avoidance Training

Rats were handled for 3-4 min each day on 5 consecutive days prior to inhibitory avoidance training. All training took place between 12:00 and 16:00 h. The inhibitory avoidance apparatus was a trough-shaped alleyway (91 cm long, 22.9 cm wide at the top, 7.6 cm wide at the bottom, and 15.2 cm deep) divided into lit (31 cm) and dark (60 cm) compartments by a sliding door that could be lowered through the floor. Each rat was placed in the lit chamber facing the door. When the rat turned completely around, the door was lowered to a height approximately 2 cm above the floor. When the rat again turned toward the door, a timer was started to record the training latency, defined as the time taken to enter (cross all four limbs into) the dark chamber. Rats that failed to enter the dark chamber within two minutes were not included in the study. Upon entering the dark chamber, the rat received a single foot shock (0.2 mA, 0.4 sec) and the door was closed to prevent reentry into the lit chamber. These training conditions were similar to those with which we have seen rapid forgetting in senescent rats and stable memory in young adult rats in past experiments (Gold et al., 1982; Morris et al., 2010; Morris et al., 2012; Sternberg et al., 1985). In these past experiments, memory in aged rats was not impaired during the first hours after training but forgetting emerged within 1-7 days after training in aged rats, at times when young rats do not exhibit forgetting for the inhibitory avoidance experiences. Also, past tests revealed that aged Fischer-344 rats do not have higher foot shock thresholds than do young adult rats (Gold et al., 1982). Thus, the conditions used in the present experiment were appropriate for tests of the neurobiological concomitants of rapid forgetting in aged rats.

For the microinfusion experiments, rats received immediate post-training infusions of artificial cerebral spinal fluid (aCSF) or glucose and were then returned to

the holding cage. Memory was assessed by measuring latencies (max of 600 sec) on a test trial 7 days later using the same procedure as above, but without the foot shock. For the immunohistochemistry experiments, rats received an immediate post-training subcutaneous injection of saline (0.9%), glucose (250 mg/kg), or epinephrine (0.1 mg/kg) and were then returned to the holding cage. To measure CREB and phosphorylated CREB (pCREB) levels, rats were euthanized 30 min after training and immunostaining procedures were used at a later time. There are differences across studies in the optimal time to assess maximal pCREB responses to training. The 30-min time point was chosen based on prior work showing robust increases in CREB phosphorylation in young adult rats at that time under similar training conditions (Morris and Gold, 2012a). We also hypothesized that 30 min would allow sufficient time for post-training treatments to enhance cell signaling processes related to CREB phosphorylation.

4.2.4. Microinfusions

Immediately following training, microinfusion probes (Plastics One) were inserted through the guide cannulae to a point 1 mm below the guide cannulae tips. A solution of aCSF (128 mM NaCl, 2.5 mM KCl, 1.3 mM CaCl2, 2.1 mM MgCl2, 0.9 mM NaH2PO4, 2.0 mM Na2HPO4, pH 7.4) containing either 1.0 or 33.4 mM glucose was infused bilaterally into the dorsal hippocampus at a rate of 0.25 µl / min for 2 min. The 1.0 mM glucose concentration in control aCSF is based on evidence that this level is seen at baseline in hippocampal CSF (McNay and Gold, 1999). Infusion probes were left in place for an additional 1 min to allow solutions to diffuse away from the probe tips. In contrast to glucose, epinephrine does not cross readily from blood to brain and neither

circulating nor central epinephrine appears to act at adrenergic receptors in the hippocampus. Therefore, intrahippocampal injections of epinephrine were not tested here.

4.2.5. Perfusion and Brain Slicing

Rats were deeply anesthetized with an overdose i.p. injection of sodium pentobarbital (Sigma-Aldrich, St. Louis, MO) and then perfused intracardially with 80 ml of 0.1 M phosphate-buffered saline (PBS) followed by 80 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Rats were decapitated and the brains were removed and placed into 4% paraformaldehyde in 0.1 M PB for ~72 hrs. The brains were transferred to 20% glycerol in 0.1 M PBS for ~48 hrs. Frozen sections (40 µM) were collected at -30o C with a Leica 1800 cryostat (Leica Microsystems, Wetzlar, Germany). For the immunohistochemistry experiments, slices through the dorsal hippocampus were collected and stored in a cryopreservative solution (250 mM 40 KD polyvinylpyrrolidone, 880 mM sucrose, 30% v/v ethylene glycol, 50 mM sodium phosphate) at -20^{o} C. For the microinfusion experiments, sections containing the guide cannulae tracts were mounted on slides, dried, stained with cresyl violet, and visualized under a light microscope. Behavioral data were discarded for those rats with probe sites outside of the dorsal hippocampus.

4.2.6. Immunohistochemistry

All steps took place at room temperature. All reactions were performed in duplicate, using alternating slices for pCREB and CREB staining ~3.3 mm posterior to bregma. Slices were washed three times for 10 min each time in 0.05 M PBS initially

and in between all subsequent steps. Slices were first incubated in blocking solution (1% H_2O_2, 1% normal goat serum (NGS), 0.02% triton x-100, 0.05 M PBS) for 10 min. They were transferred to a pre-incubation solution (2% NGS, 0.4% triton x-100, 0.05 M PBS) for 20 min and then incubated overnight in a solution (1% NGS, 0.4% triton x-100, 0.05 M PBS) containing a rabbit primary antibody for Ser-133 phosphorylated CREB or total CREB (Millipore, Billerica, MA) diluted 1:4000. The next day, the slices were placed for 1 hr in a solution (1% NGS, 0.2% triton x-100, 0.05 M PBS) containing a goat anti-rabbit biotinylated secondary antibody (Santa Cruz, Santa Cruz, CA). They were next incubated for 30 min with ABC reagent (Vector, Burlingame, CA) in 0.05 M PBS, followed by incubation with DAB substrate (Vector) for 4 min. Slices were mounted onto slides and allowed to dry overnight. The next morning, slices were dehydrated with a graded ethanol series of washes, then coverslipped using DPX mountant (Sigma-Aldrich).

4.2.7. Image Acquisition and Analysis

Sections were imaged using a Leica DM 6000B/CTR6000 light microscope and a Leica DFC350 FX video camera, which was interfaced to a PC computer. This system was used in conjunction with Image-Pro software (Media Cybernetics, Inc., Bethesda, MD) for image acquisition and to correct for unevenness in illumination across images. Image J software (NIH, Bethesda, MA) was used to quantify the optical density of pCREB and CREB staining. The six brain regions analyzed were the dentate gyrus and areas CA3 and CA1 of the hippocampus, the basolateral and lateral nuclei of the amygdala, and the piriform cortex. The hippocampus and amygdala regions were selected because of prior evidence that training, as in the present experiment, elicited

clear phosphorylation of CREB in young rats but not in old rats. The piriform cortex was selected as a control region based on evidence that CREB phosphorylation is not altered by age or by training in either young or old rats (Morris and Gold, 2012a). Morris and Gold (2012a) provides an illustration of the specific targeted areas as well as a detailed discussion of CREB activation in these brain regions. The auto-thresholding method in Image J was used to ensure that only specifically labeled cells were being measured. For each image, the optical density of a nearby region with no or little specific staining was calculated and used for background subtraction.

4.2.8. Statistical Analyses

All analyses were performed using Statview software. Behavioral training and retention latencies were analyzed using a non-parametric Kruskal-Wallis one-way analysis of variance, followed by Mann-Whitney tests for individual comparisons, if appropriate. The immunohistochemistry experiments were run in two cohorts. In the first cohort, rats were trained on the inhibitory avoidance task with a post-training injection of saline or glucose. These groups were compared to untrained rats that were handled but not trained on the task. In the second cohort, rats were trained with a post-training injection of saline or epinephrine, and these groups were also compared to untrained rats. To facilitate comparison between glucose and epinephrine, the data from the two cohorts were analyzed together by normalizing to the values obtained in the untrained rats. The optical densities of CREB and pCREB immunostaining, as well as pCREB:CREB ratios, were analyzed using two-way ANOVAs (age x training/treatment group) with post hoc Fisher PLSD tests where appropriate.

4.3. RESULTS

4.3.1. Inhibitory Avoidance Training with Intrahippocampal Glucose Injections.

Figure 4.1 shows training and 7-day retention latencies in young and old rats following inhibitory avoidance training and post-training intrahippocampal microinfusions of aCSF (Ns = 7 and 9 for young and old rats, respectively) or glucose (Ns = 8 and 11 for young and old rats, respectively). Kruskal–Wallis analyses of variance revealed a significant group effect on retention latencies ($p < .01$) but not on training latencies. Post-hoc Mann-Whitney U-tests showed that the old rats injected with glucose had significantly higher 7-day retention latencies compared to those of old rats that had received aCSF injections ($p < .05$). The higher latencies seen in the old rats treated with glucose were similar to those of young rats, with the group medians reaching the ceiling 600 sec maximum in each case. Figure 4.2 illustrates infusion needle tip placements in the dorsal hippocampus for young and old rats.

4.3.2. Inhibitory Avoidance Training for Immunohistochemistry Experiments.

For the immunohistochemistry experiments, retention testing latencies were not assessed since rats were euthanized 30 min after training. There were no significant differences in training latencies; the medians ranged from 5.5 – 7 sec across all groups (data not shown). Two old rats failed to cross into the dark chamber within two minutes and were not included in the study. Only two other rats had training latencies greater than 20 sec (40 and 120 sec in the young epinephrine and old glucose groups, respectively).

4.3.3. CREB and pCREB Immunostaining Following Training

Immunostaining levels of pCREB and total CREB, as well as pCREB:CREB ratios, were examined in young and old rats 30 min after inhibitory avoidance training. Rats were analyzed in four treatment groups: untrained rats (Ns = 16 for both young and old) and trained rats receiving post-training injections of saline (Ns = 16), epinephrine (Ns = 8), or glucose (Ns = 8).

4.3.3.1. Hippocampus: Dentate Gyrus.

Figure 4.3 (A and B) shows the results for pCREB immunostaining in the dentate gyrus region of the hippocampus. A two-way ANOVA revealed a main effect of training/treatment on pCREB staining ($F_{(3,88)} = 7.51$, $p < .001$). In young rats, training increased pCREB staining as compared to values in untrained rats. The increase was evident in all three trained groups that received post-training injections of saline, epinephrine, or glucose (ps < .05 vs. untrained where ps refer to the results of post-hoc comparisons). In old rats, training did not result in increased pCREB staining, as evident in the comparison of trained rats that received saline vs. untrained rats. Of particular interest here is the finding that post-training administration of glucose and epinephrine in aged rats significantly enhanced pCREB staining compared to both the untrained and saline groups (ps < .05).

There was a main effect of age ($F_{(1,88)} = 7.90$, $p < .01$) on CREB immunostaining in the dentate gyrus (Figure 4.3C). However, post-hoc tests revealed no significant differences between young and old untrained rats. Neither training nor treatments with epinephrine or glucose significantly altered CREB staining in young or old rats ($F_{(3,88)} = 0.31$, $p > 0.2$).

There was a main effect of training/treatment ($F_{(3,88)} = 8.60$, $p < .0001$) on pCREB:CREB ratios in the dentate gyrus (Figure 4.3D). In young rats, training resulted in increased pCREB:CREB ratios, with higher pCREB:CREB ratios in rats receiving post-training injections of saline, epinephrine, or glucose (ps < .01 vs. untrained). However, training had no effect in old rats, as evident by similar pCREB:CREB ratios between untrained rats and those receiving post-training saline injections. Interestingly, post-training administration of glucose but not epinephrine led to increased pCREB:CREB ratios compared to both untrained and saline-injected rats (ps < .01).

4.3.3.2. Hippocampus: Area CA3.

In contrast to the results seen in dentate gyrus, there were no effects of training or treatments with epinephrine or glucose on pCREB staining ($F_{(1,88)} = 2.99$, $p > 0.2$; Figure 4.4A and 4.4B) or on pCREB:CREB ratios ($F_{(3,88)} = 0.10$, $p > 0.2$; Figure 4.4D) in area CA3. CREB staining in area CA3 decreased with age ($F_{(1,88)} = 24.21$, $p < .0001$), with significantly lower levels in old compared to young untrained rats ($p < .0001$), but did not vary by training or treatments ($F_{(3,88)} = 0.23$, $p > 0.2$; Figure 4.4C).

4.3.3.3. Hippocampus: Area CA1.

The results for area CA1 (Figure 4.5) were very similar to those for the dentate gyrus, and thus are not presented in detail here. The major exception was in the pCREB results for old rats. Specifically, glucose significantly enhanced pCREB staining compared to both the untrained and saline groups (ps < .05). Epinephrine was less effective, significantly enhancing pCREB staining compared to untrained ($p < .05$) but not saline-injected rats.

4.3.3.4. Basolateral Amygdala.

Figure 4.6 (A and B) shows the results for pCREB immunostaining in the basolateral nucleus of the amygdala. There was a main effect of training/treatment on pCREB staining ($F_{(3,88)} = 5.87$, $p < .01$). In young rats, training significantly enhanced pCREB staining; the trained groups that received saline, glucose or epinephrine all had higher pCREB staining compared to untrained rats (ps < .01). Epinephrine and glucose did not enhance pCREB levels beyond those attained after training plus saline. In old rats, the trained saline rats had slightly higher levels of pCREB expression than did untrained rats, but this was not a significant difference. Interestingly, in old rats, epinephrine but not glucose augmented pCREB expression to levels significantly above those of untrained rats ($p < .05$).

There was a main effect of age ($F_{(1,88)} = 21.41$, $p < .0001$) on CREB immunostaining, with significantly lower CREB staining in old compared to young untrained rats ($p < .05$; Figure 4.6C). However, neither training nor treatments significantly altered CREB staining in young or old rats ($F_{(3,88)} = 0.23$, $p > 0.2$).

The results obtained with pCREB:CREB ratios (Figure 4.6D) were similar to those seen with pCREB alone. There was a main effect of training/treatment ($F_{(3,88)} = 7.04$, $p < .001$). Compared to the ratios of untrained young rats, pCREB:CREB ratios increased with training followed by saline, glucose or epinephrine (ps < 01). In old rats, the ratios also increased above untrained values but only significantly so for the epinephrine-treated group ($p < .05$), though there was a trend toward enhancement in the glucose group ($p < .06$).

4.3.3.5. Lateral Amygdala.

The results in the lateral amygdala (data not shown) were very similar to those in the basolateral amygdala for pCREB, CREB, and pCREB:CREB ratios, and are thus not presented in detail here.

4.3.3.6. Piriform Cortex.

There were no main effects of age ($Fs_{(1,88)} < 0.52$), training, or treatment ($Fs_{(3,88)} < 0.09$) on pCREB, CREB, or pCREB:CREB ratios in the piriform cortex (data not shown).

4.3.3.7. Summary of Immunohistochemistry Results.

Table 4.1 summarizes the effects of epinephrine and glucose on modulating CREB phosphorylation across brain regions.

4.4. DISCUSSION

4.4.1. Age-Related Memory Impairments and Reversal by Intrahippocampal Glucose

There are two main behavioral findings reported here. The first is that memory was impaired in old rats relative to young rats at 7 days after inhibitory avoidance training. These findings replicate past results, which showed that age-related impairments in memory after training on inhibitory avoidance training emerge with time (Gold et al., 1982; Zornetzer et al., 1982), i.e. old rats forget more rapidly than do young rats. Of note, differences in perception of the foot shock do not account for the findings (Foster and Kumar, 2007; Frye et al., 2010; Gold et al., 1982; Morris et al., 2010). The training conditions utilized in the present studies are the same as those for which systemic administration of epinephrine or glucose enhances memory (Morris et al.,

2010; Sternberg et al., 1985). Thus, the training conditions used to evaluate CREB activation were appropriate for showing age-related impairments in memory and in modulation of memory.

The second main behavioral finding is that post-training injections of glucose directly into the hippocampus enhance later memory in aged rats. When drugs are administered after training, as here, effects on later memory for the learned experience cannot be attributed to alterations in non-mnemonic factors such as sensory, motor, or motivational variables (Gold, 2008; Gold and Korol, 2010; McGaugh, 1989, 2000). These results complement our previous findings that systemic epinephrine or glucose administration can enhance memory in aged animals in several settings, including when administered after training in both rodents (Morris et al., 2010; Sternberg et al., 1985) and humans (Manning et al., 1992).

To our knowledge, this is the first example of a direct brain injection enhancing memory in aged rats using a post-training design. Previous work in young rats has shown that direct infusions of glucose into the lateral ventricles or into specific brain areas, including the medial septum, hippocampus, and amygdala, enhance memory (Canal et al., 2005; Pych et al., 2006; Ragozzino et al., 1996, 1998; Schroeder and Packard, 2003; Stefani and Gold, 1998; Stefani et al., 1999). Together with the present results, these findings are consistent with the idea that glucose acts directly in the brain, rather than in the periphery, to enhance memory in young and old rats.

4.4.2. Age-Related Differences in CREB and pCREB Expression at Baseline and in Response to Training.

Compared to levels in young rats, baseline CREB levels were significantly depressed in area CA3 and in the basolateral and lateral amygdala of old compared to young untrained rats. The age-related decreases in baseline CREB levels were not evident in area CA1, the dentate gyrus, or piriform cortex. These results replicate those we described previously, where age-related decreases in CREB at baseline were evident in area CA3 and the amygdala, but not other brain regions tested (Morris and Gold, 2012a). More generally, age-related decreases in CREB expression observed here are also consistent with the findings of previous studies (Brightwell et al., 2004; Countryman and Gold, 2007; Trofimiuk et al., 2010).

In addition to decreases in CREB expression in some brain areas, there were also significant age-related differences in CREB phosphorylation in response to training. In young rats, training with a post-training saline injection enhanced CREB phosphorylation compared to untrained controls. The increased pCREB values after training were region-specific, occurring in the dentate gyrus, area CA1, and the basolateral and lateral amygdala, but not in area CA3 or the piriform cortex. This same pattern of anatomical localization of CREB activation was seen previously in response to training without post-training injection of saline or other treatment (Morris and Gold, 2012a). Thus, it appears that the pCREB activation after training observed here in the saline controls is a response to training and not to the stress of injection.

In contrast to the results seen in young rats, pCREB levels in old rats were not responsive to training in any brain region tested. These results match those described previously, in which there were age-related deficits in CREB phosphorylation in the hippocampus and amygdala in response to training (Morris and Gold, 2012a). The

findings are also similar to the age-dependent deficits in hippocampal CREB activation and associated memory impairments after training on other hippocampus-sensitive tasks (Countryman and Gold, 2007; Kudo et al., 2005; Monti et al., 2005; Porte et al., 2008a; Xu et al., 2010). Together, these results suggest that old rats are deficient in their ability to activate CREB in the hippocampus and amygdala in response to training. These are brain regions associated with inhibitory avoidance learning and modulation of that learning (Cammarota et al., 2008; Canal and Gold, 2007; Izquierdo et al., 1992, 2002; Jobim et al., 2012; McGaugh et al., 2002; McIntyre et al., 2005; McReynolds et al., 2010; Milekic et al., 2007; Rossato et al., 2004). Using compromised CREB activation as a measure of functional integrity, the hippocampus and amygdala are therefore brain regions in which function impaired by age may contribute to the rapid forgetting seen in aged rats.

4.4.3. Modulation of CREB Phosphorylation by Glucose and Epinephrine.

In young rats, neither epinephrine nor glucose resulted in activation of CREB in the hippocampus or amygdala beyond the levels produced by training alone. Apparently, then, in young rats, the foot shock used during training was adequate to increase pCREB to levels that were not sensitive to further enhancement by these modulators of memory formation. The findings that a single foot shock was sufficient to increase pCREB expression in the hippocampus and amygdala are comparable to findings reported by others after inhibitory avoidance training (Bernabeu et al., 1997; Cammarota et al., 2000; Izquierdo et al., 2002; Taubenfeld et al., 1999; Viola et al., 2000) and after training in other avoidance tasks (Bilang-Bleuel et al., 2002; Impey et al., 1998; Kogan and Richter-Levin, 2008; Porte et al., 2008b; Stanciu et al., 2001;

Trifilieff et al., 2006). The question of whether activation of CREB is associated with enhancement of memory in young rats was not addressed in the present experiments. Note that the training conditions used here produced maximal memory scores (600 sec) in young rats with or without treatments. Because a ceiling effect interfered with demonstrations of possible enhancement of memory, the experimental conditions did not permit assessment of the possibility that modulators of memory might activate CREB in a manner associated with enhancement of memory. Although these treatments did not augment CREB activation beyond that attained with training alone in young rats, it will be important to determine whether CREB activation plays a role in enhancement of memory with epinephrine or glucose in young rats.

In old rats, pCREB responses to training per se were not evident in the hippocampus or amygdala. However, treatment with either epinephrine or glucose augmented those responses under conditions where the treatments would enhance memory (Morris et al., 2010; Sternberg et al., 1985). While both epinephrine and glucose enhance memory in aged rats, the effects are generally more robust with glucose than with epinephrine (Morris et al., 2010). The pCREB results in the dentate gyrus and CA1 region of the hippocampus reflect this best. In these brain regions, glucose was somewhat more effective than was epinephrine at augmenting CREB activation. These findings suggest that activation of CREB in the hippocampus parallels and may contribute to memory enhancement by epinephrine and glucose in aged rats.

Related evidence indicates that glucose augments training-related release of acetylcholine in the hippocampus of young adult rats (Gold, 2003; Kopf et al., 2001; Morris et al., 2010; Ragozzino and Gold, 1995; Ragozzino et al., 1996, 1998) and is

more effective than epinephrine at increasing acetylcholine release during training in the hippocampus of aged rats (Morris et al., 2010). Thus, one possibility is that glucose enhances CREB phosphorylation through activation of the cholinergic system. Related to this possibility is evidence that fimbria-fornix lesions, which disrupt cholinergic innervation to the hippocampus (Erb et al., 1997; Nilsson and Björklund, 1992), also disrupt hippocampal CREB activation and impair memory for inhibitory avoidance (Taubenfeld et al., 1999, 2001). Acetylcholine can result in phosphorylation of CREB through binding to muscarinic 3 (M_3) receptors (Greenwood and Dragunow, 2002, 2010) or nicotinic α7 receptors (Bitner et al., 2007, 2010; Gubbins et al., 2010; Tietje et al., 2008). Actions mediated by α7 receptors are somewhat more likely because these receptors are highly expressed in the hippocampus (Séguéla et al., 1993; Tribollet et al., 2004), while M_3 receptors are expressed at low levels in the hippocampus (Buckley et al., 1988; Levey et al., 1994; Vilaró et al., 1993).

While post-training injections of either epinephrine or glucose increased pCREB activation in old rats, only epinephrine did so significantly in the amygdala. Thus, the rank order of efficacy of epinephrine and glucose in augmenting pCREB levels in the amygdala does not match their effects on memory (Morris et al., 2010). These findings suggest that activation of the hippocampus may contribute more than does activation of the amygdala to the enhancement of memory in old rats by epinephrine and glucose. Epinephrine, like glucose, may enhance CREB phosphorylation through activation of the cholinergic system. However, because epinephrine is less effective than glucose at enhancing acetylcholine release in the hippocampus of old rats, epinephrine may work through additional mechanisms to activate CREB. Particularly in the amygdala,

epinephrine may enhance CREB phosphorylation through the activation of neurotransmitters other than acetylcholine, such as norepinephrine. Noradrenergic mechanisms in the amygdala appear to be important for memory modulation generally and for enhancement of memory by epinephrine in particular (Ferry and McGaugh, 2000; McGaugh, 2004; McGaugh et al., 2002; McIntyre et al., 2003). In young adult rats, epinephrine or foot shock produces rapid and sustained increases in norepinephrine release in the amygdala (Canal et al., 2008; Galvez et al., 1996; Gold and van Buskirk, 1978a,b; McIntyre et al., 2002; McReynolds et al., 2010; Quirarte et al., 1998; Williams et al., 1998). In addition, injections of adrenergic antagonists into the amygdala attenuate the memory-enhancing effects of epinephrine (Liang et al., 1986, 1990; McIntyre et al., 2005; McReynolds et al., 2010; Williams and McGaugh, 1993). Importantly, it is not likely that noradrenergic signaling mediates enhancement of memory by glucose. Peripheral glucose administration prior to spontaneous alternation testing significantly improved working memory scores but had no effect on hippocampal norepinephrine release (Men et al., 1999). In contrast, glucose significantly enhanced hippocampal acetylcholine release and improved memory under similar and related conditions (Ragozzino et al., 1996, 1998; Stefani and Gold, 2001). Noradrenergic signaling in the amygdala and other brain areas leads to activation of cAMP-mediated signaling pathways, including CREB phosphorylation (Barros et al., 1999; Chen et al., 2007; Davies et al., 2004; Ferry et al., 1999; Patel et al., 2010; Yuan et al., 2000). These findings suggest that epinephrine, unlike glucose, may modulate CREB phosphorylation by increasing norepinephrine release in the amygdala. This hypothesis fits well with the current results, in which epinephrine significantly enhanced CREB

phosphorylation in the amygdala of old rats, presumably without associated increases in blood glucose. However, examining norepinephrine release in the amygdala and hippocampus of old rats is an important future direction.

4.4.4. Conclusions.

The present experiments support the hypothesis that age-related deficits in blood glucose responses to endogenous epinephrine release may alter neurobiological processes related to memory formation. In old rats, increases in circulating epinephrine in response to training or stress may represent an impaired physiological mechanism in the liver for producing increases in blood glucose levels. The alterations in this neuroendocrine response could change the relative contributions of neurotransmitters and memory systems involved in cognitive processing. Although both epinephrine and glucose can support improved memory in old rats, both peripheral and intrahippocampal glucose administration are particularly effective in this regard. Thus, examining the neural mechanisms underlying glucose-mediated memory processes may provide greater insight into strategies for reversing age-related cognitive decline.

4.5. FIGURES

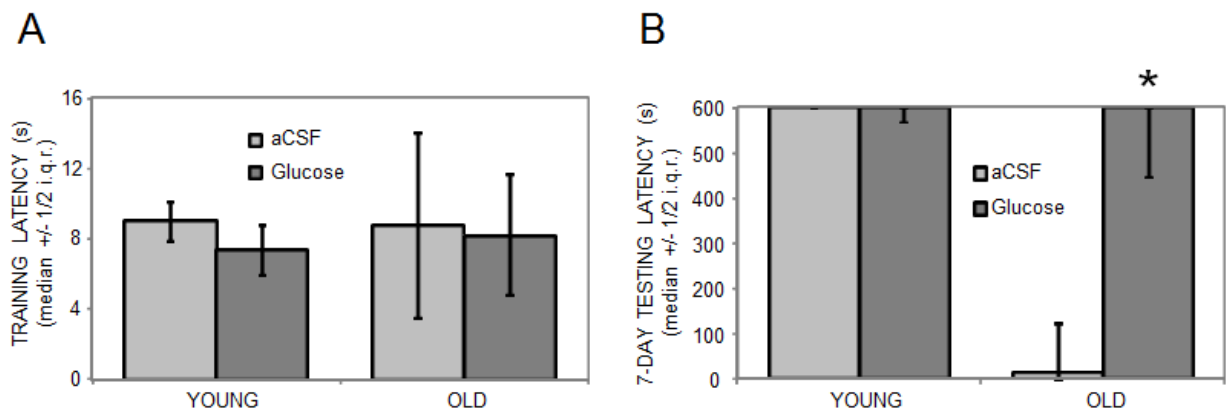

Figure 4.1. Effects of age and intrahippocampal glucose administration on 7-day inhibitory avoidance retention latencies.

(A) There were no significant differences in training latencies across groups. (B) Old rats receiving post-training intrahippocampal injections of aCSF had significantly lower 7-day retention latencies compared to young rats receiving either aCSF or glucose injections. Post-training glucose injections reversed age-related impairments, improving retention latencies in old rats to levels seen in young rats. (*) $p < .05$ vs. old aCSF group. i.q.r. = interquartile range.

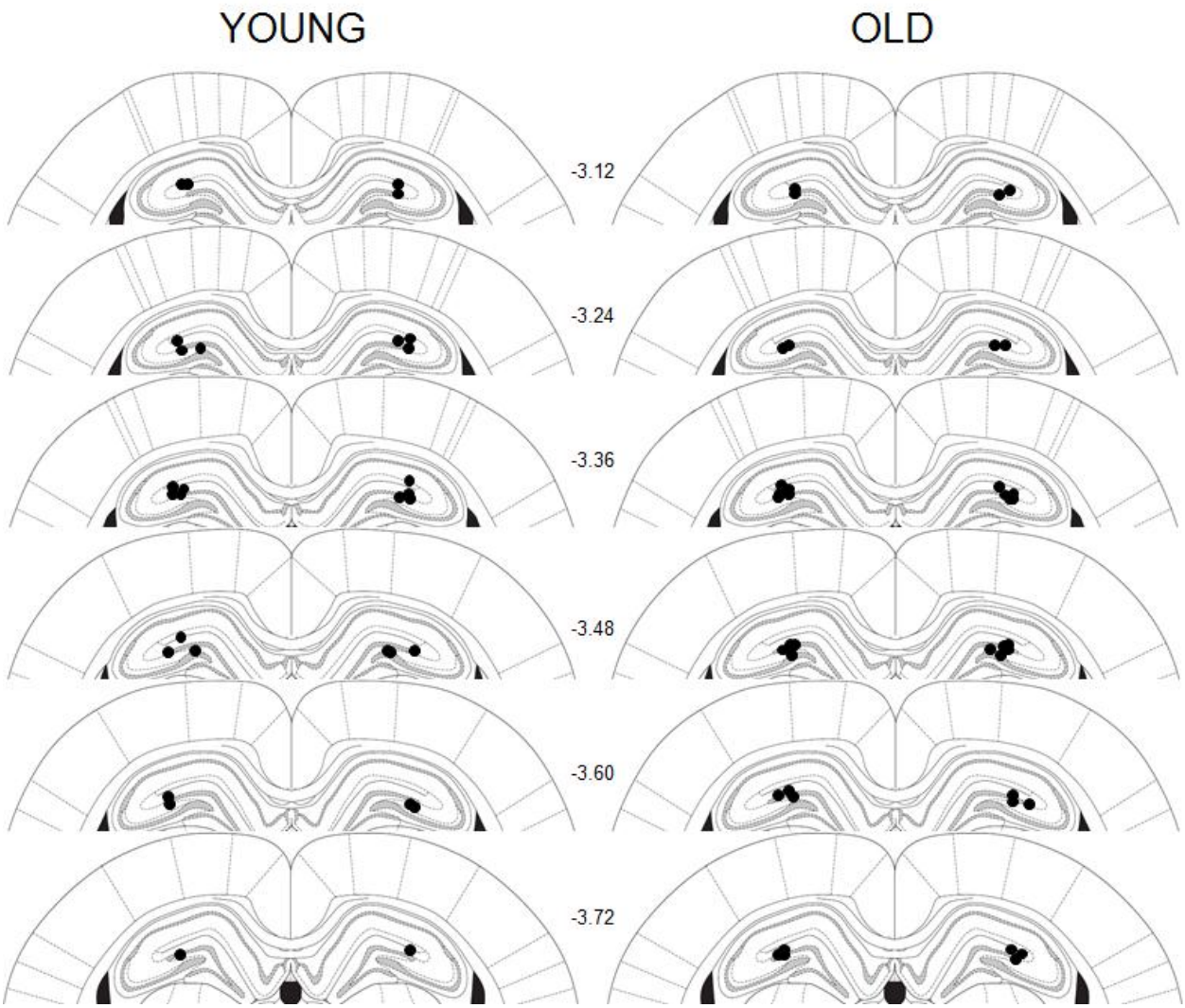

Figure 4.2. Infusion sites targeting the dorsal hippocampus in young and old rats.

Filled circles represent tips of infusion tracts. Numbers refer to distance in mm posterior to bregma. Adapted with permission from Paxinos and Watson (2005).

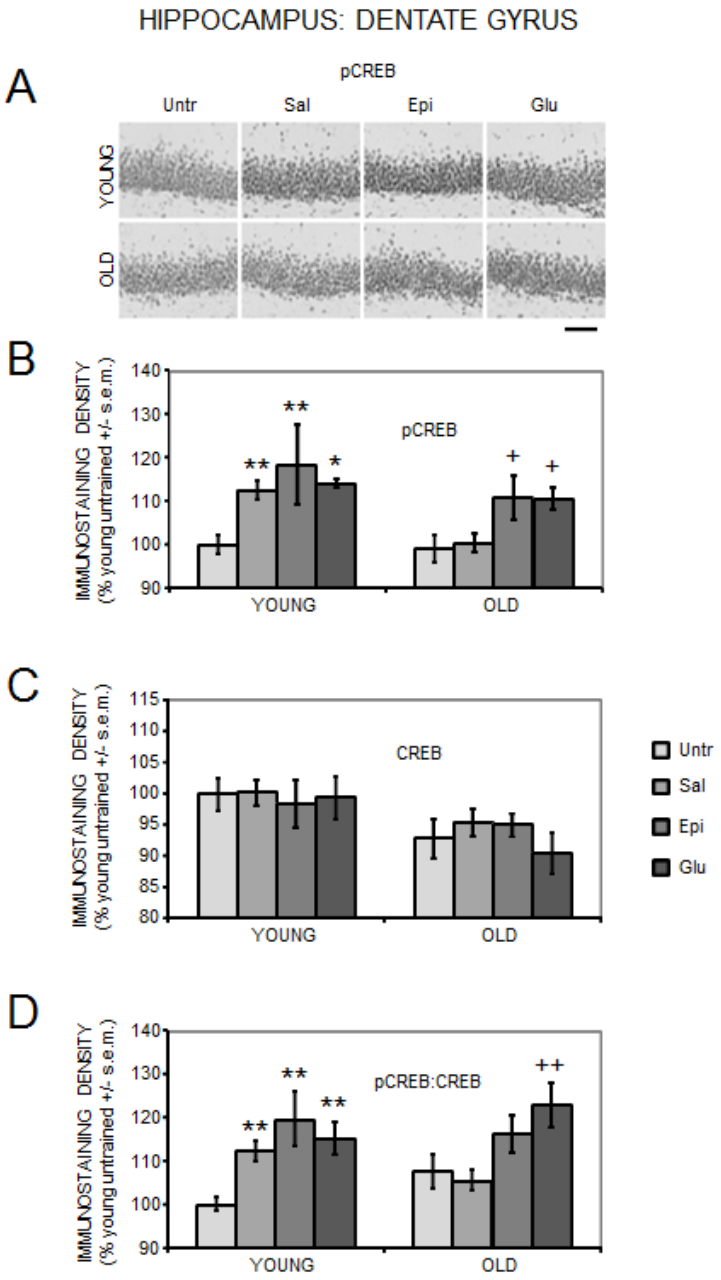

Figure 4.3. Age-, training-, and treatment-associated differences in pCREB and CREB immunoreactivity in the dentate gyrus of the hippocampus.

(A) Representative photomicrographs of pCREB immunostaining. Scale bar = 100 microns. (B) Old rats had training-related deficits in pCREB activation, which were attenuated by epinephrine and glucose. (*) $p < .05$ vs. young untrained group. (**) $ps < .01$ vs. young untrained group. (+) $ps < .05$ vs. old untrained and saline groups. (C) There were significantly lower CREB levels in old compared to young rats. (D) Old rats had training-related deficits in pCREB:CREB ratios, which were attenuated by glucose. (**) $ps < .01$ vs. young untrained group. (++) $ps < .01$ vs. old untrained and saline groups.

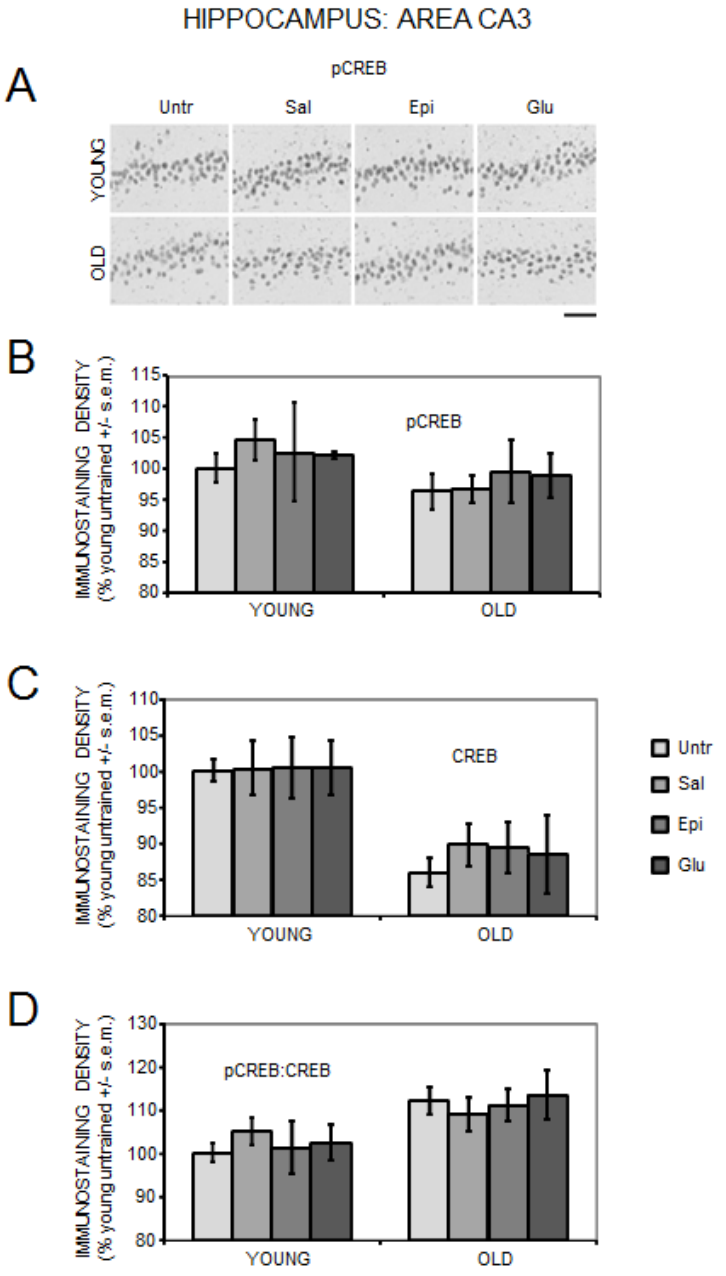

Figure 4.4. Age-, training-, and treatment-associated differences in pCREB and CREB immunoreactivity in area CA3 of the hippocampus.

(A) Representative photomicrographs of pCREB immunostaining. Scale bar = 100 microns. (B) There were no significant age-, training-, or treatment-related differences in pCREB levels. (C) There were significantly lower CREB levels in old compared to young rats. (D) pCREB:CREB ratios were significantly elevated in old compared to young rats.

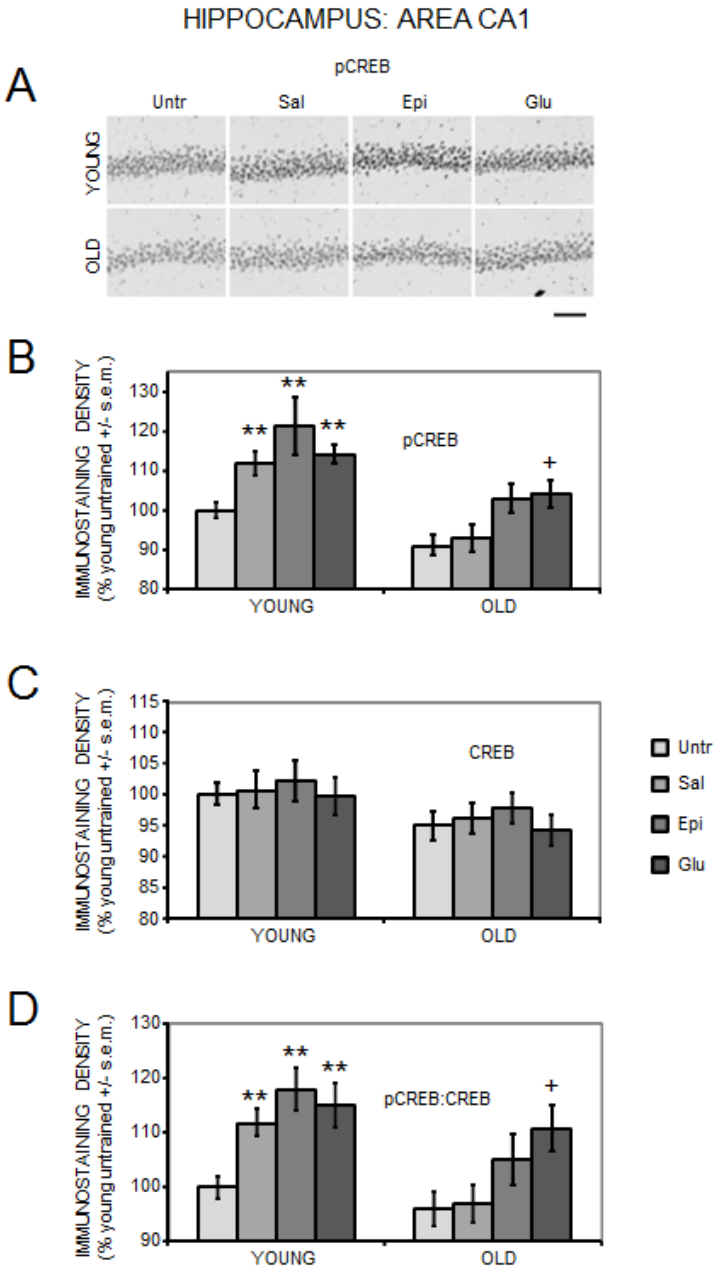

Figure 4.5. Age-, training-, and treatment-associated differences in pCREB and CREB immunoreactivity in area CA1 of the hippocampus.

(A) Representative photomicrographs of pCREB immunostaining. Scale bar = 100 microns. (B) Old rats had training-related deficits in pCREB activation, which were attenuated by epinephrine and glucose. (**) ps < .01 vs. young untrained group. (+) ps < .05 vs. old untrained and saline groups. (C) There were significantly lower CREB levels in old compared to young rats. (D) Old rats had training-related deficits in pCREB:CREB ratios, which were attenuated by glucose. (**) ps < .01 vs. young untrained group. (+) ps < .05 vs. old untrained and saline groups.

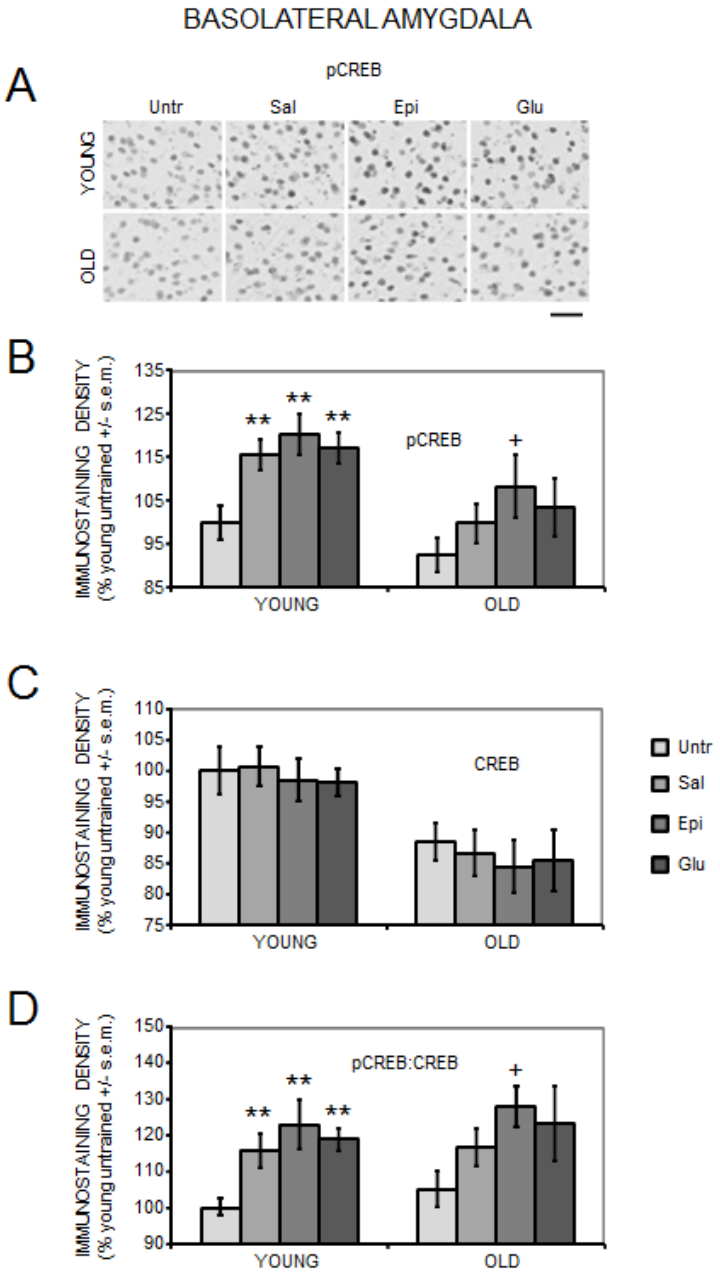

Figure 4.6. Age-, training-, and treatment-associated differences in pCREB and CREB immunoreactivity in the basolateral nucleus of the amygdala.

(A) Representative photomicrographs of pCREB immunostaining. (B) Old rats had training-related deficits in pCREB activation, which were attenuated by epinephrine. (**) ps < .01 vs. young untrained group. (+) p < .05 vs. old untrained group. Scale bar = 50 microns. (C) There were significantly lower CREB levels in old compared to young rats. (D) Old rats had training-related deficits in pCREB:CREB ratios, which were attenuated by epinephrine. (**) ps < .01 vs. young untrained group. (+) p < .05 vs. old untrained group.

Table 4.1. Effects of epinephrine and glucose on modulating training-related CREB phosphorylation.

	OLD RATS	
	EPINEPHRINE	**GLUCOSE**
pCREB		
DG	✓	✓
CA3		
CA1	✓	✓
BLA	✓	
LA	✓	
PIR		
pCREB:CREB		
DG		✓
CA3		
CA1		✓
BLA	✓	
LA	✓	
PIR		

✓denotes significant effect versus untrained rats. Brain regions analyzed were dentate gyrus (DG), area CA3, and area CA1 of the hippocampus, basolateral (BLA) and lateral (LA) amygdala, and piriform cortex (PIR).

CHAPTER 5: GLUCOSE ATTENUATES IMPAIRMENTS IN MEMORY AND CREB ACTIVATION PRODUCED BY AN α4β2 BUT NOT AN α7 NICOTINIC RECEPTOR ANTAGONIST

Glucose improves memory for a variety of tasks when administered to rats and mice near the time of training. Prior work indicates glucose may enhance memory by increasing the synthesis and release of the neurotransmitter acetylcholine in the brain. To investigate if specific acetylcholine receptor subtypes may mediate some of the memory-enhancing actions of glucose, we examined the effects of subtype-specific nicotinic acetylcholine receptor antagonists on memory in Fischer-344 rats and also examined the ability of glucose to reverse drug-induced impairments. Pre-training peripheral injections of methyllycaconitine (MLA) or dihydro-beta-erythroidine (DHβE), which are specific α7 and α4β2 nicotinic receptor antagonists, respectively, dose-dependently impaired retention latencies in an inhibitory avoidance task when tested 7-days but not 1 hour after training. Immediate post-training glucose injections attenuated the impairments, but were more effective in attenuating the DHβE-induced impairments. Likewise, peripheral or direct intrahippocampal injections of MLA or DHβE dose-dependently impaired alternation spatial working memory scores on a spontaneous alternation task. Concurrent administration of glucose reversed DHβE- but not MLA-induced impairments. CREB phosphorylation downstream of cholinergic signaling was assessed 30 minutes after spontaneous alternation testing with intrahippocampal drug infusions. Both MLA and DHβE impaired hippocampal CREB phosphorylation, with glucose reversing DHβE but not MLA-induced deficits. The effectiveness of glucose in reversing DHβE- but not MLA- induced impairments in behavioral performance and

CREB phosphorylation suggests that activation of α7 receptors may play an important role in memory enhancement by glucose.

5.1. INTRODUCTION

In humans and rodents, rises in blood glucose in response to stressful or emotional stimuli mediate memory improvement for the associated events. Likewise, glucose administration prior to or shortly after an event can increase its salience, converting a relatively unimportant event to a memorable one (Gold and Korol, 2010; Korol and Gold, 1998, 2007; Messier, 2004). In young adult rodents, increases in blood glucose enhance memory in a wide variety of behavioral tasks, ranging from tests of spatial working memory to assessments of memory at long intervals after training (cf. Gold, 2008). Glucose can also reverse age-related memory impairments in these same tasks, improving performance in old rats to the levels seen in young rats (Gold, 2005; McNay and Gold, 2001; Morris et al., 2010; Salinas and Gold, 2005).

Glucose is thought to work directly in the brain to enhance cellular and molecular memory processes, perhaps mediated by enhanced aerobic glycolysis and lactate production in astrocytes (Newman et al., 2011; Suzuki et al., 2011). Previous work suggests that systemically- or centrally-administered glucose may enhance memory by augmenting central release of the neurotransmitter acetylcholine. A number of microdialysis studies show that both peripheral and direct intrahippocampal glucose administration augment training-related acetylcholine release in the hippocampus, concurrent with memory enhancement (Kopf et al., 2001; Morris et al., 2010; Ragozzino and Gold, 1995; Ragozzino et al., 1996, 1998). These effects on acetylcholine release appear to be regionally specific and do not occur in animals at rest (Ragozzino et al.,

1996, 1998), suggesting that glucose supports enhanced acetylcholine release in particular brain areas where it is needed to facilitate memory processes.

An open question is whether the memory-improving actions of glucose rely on signaling through some acetylcholine receptor subtypes more than others. Acetylcholine activates both muscarinic and nicotinic acetylcholine receptors. The subtypes of these receptors have diverse functions and variable expression patterns throughout the brain. Previous work indicates that peripheral injections of general muscarinic and nicotinic antagonists impair performance in a number of behavioral memory paradigms, including inhibitory avoidance and spontaneous alternation tasks, and that co-administration of glucose attenuates these impairments (Blanchard and Duncan, 1997; Kopf and Baratti, 1994; Ragozzino and Gold, 1991; Ragozzino et al., 1994a; Stone et al., 1988, 1991, 1995). These results imply a lack of regional and receptor specificity in glucose's actions, suggesting that glucose could compensate for deficits in muscarinic receptors by promoting signaling through nicotinic receptors, and vice versa. However, the results are difficult to interpret because of the wide-ranging effects of these drugs throughout the body and brain, including effects not specific to memory processes.

The emergence of drugs targeting specific acetylcholine receptor subtypes in the brain, as opposed to general muscarinic or nicotinic receptors, makes it possible to begin evaluating which receptor subtypes may be important to the memory-enhancing effects of glucose. Two nicotinic receptor subtypes, α4β2 and α7, possess the functional properties and regional distribution that make them potential mediators of glucose's effects (Cincotta et al., 2008; Graef et al., 2011; Kenney and Gould, 2008; Levin et al., 2006). Neuronal nicotinic receptors are ionotropic receptors composed of various

combinations of five membrane-spanning subunits. Nine α (α2–α10) and three β (β2–β4) subunits have been identified, making a large number of receptor subtype combinations possible. However, the heteromeric α4β2 and homomeric α7 receptor subtypes have garnered the most attention due to their high levels of expression in the brain and roles in a variety of cognitive processes. In many situations, α4β2 and α7 receptors appear to function similarly. For example, a number of studies have utilized direct brain infusions of methyllycaconitine (MLA) or dihydro-β-erythroidine (DHβE), which are competitive antagonists for α7 and α4β2 receptors, respectively, to assess working memory performance in the radial arm maze. Infusions of either MLA or DHβE into the ventral or dorsal hippocampus, amygdala, or frontal cortex induced working memory impairments, with only minor dose-related differences between the drugs (Addy et al., 2003; Bettany and Levin, 2001; Chan et al., 2007; Levin et al., 2002; Nott and Levin, 2006). In other cases, there are distinct functional differences between the α4β2 and α7 receptor subtypes. For instance, there are several studies indicating a specific role for α4β2 but not α7 receptors in mediating the effects of nicotine on enhancing memory for contextual fear conditioning (Davis and Gould, 2006, 2007; Davis et al., 2007; Kenney et al., 2012).

Recent evidence indicates that glucose and nicotinic acetylcholine receptors activate a similar downstream molecular signaling pathway related to memory formation. Specifically, cAMP response element binding protein (CREB) is a transcription factor widely implicated in the formation of long-lasting memory and long-term changes in synaptic plasticity (Alberini, 2009; Benito and Barco, 2010; Carlezon et al., 2005; Morris and Gold, 2012a; Silva, 1998; Yin and Tully, 1996). A number of

studies indicate that nicotine administration activates CREB in a variety of neuronal cell types in both *in vitro* and *in vivo* models (Chang and Berg, 2001; Hu et al., 2002; Nakayama et al., 2001; Pascual et al., 2009; Tang et al., 1998). Likewise, glucose administration following inhibitory avoidance training reverses age-related memory impairments, with parallel activation of CREB phosphorylation. Consistent with the view of regional selectivity in the memory-enhancing actions of glucose, there is a high degree of regional variability in glucose-mediated increases in CREB phosphorylation, including within subregions of the hippocampus (Morris and Gold, 2012b).

The following work examines the effects of the α7 antagonist MLA and the α4β2 antagonist DHβE on memory processes in inhibitory avoidance and spontaneous alternation tasks, and also examines the ability of glucose to attenuate drug-induced impairments. The experiments utilize both peripheral and direct intrahippocampal injections, and include correlations with the expression of phosphorylated CREB (pCREB) in the hippocampus.

5.2. METHODS

5.2.1. Subjects.

Young adult (3 to 4 mo.) male Fischer-344 rats ordered from Taconic Farms (Germantown, NY) were individually housed in translucent cages with a 12-h light/dark cycle (lights on at 07:00 h) and *ad libitum* access to food and water. Animal pain and discomfort were minimized, and all experiments were conducted in accordance with animal care guidelines established by the National Institute of Health and the University of Illinois, which is fully accredited (AAALAC). Rats were handled for 2-3 min each day on 5 consecutive days prior to behavioral training.

5.2.2. Preparation of drugs.

Methyllycaconitine citrate salt hydrate (MLA) was obtained from Sigma-Aldrich (St. Louis, MO). Dihydro-β-erythroidine hydrobromide (DHβE) was obtained from Tocris Bioscience (Minneapolis, MN). MLA, DHβE, and glucose were dissolved in saline for intraperitoneal (i.p.) administration or in artificial cerebral spinal fluid (aCSF; 128 mM NaCl, 2.5 mM KCl, 1.3 mM CaCl2, 2.1 mM MgCl2, 0.9 mM NaH2PO4, 2.0 mM Na2HPO4, 1 mM glucose, pH 7.4) for intrahippocampal administration. The 1.0 mM glucose concentration in the aCSF is based on evidence that this level is seen at baseline in hippocampal extracellular fluid (McNay and Gold, 1999).

5.2.3. Surgery and intrahippocampal drug infusions.

Rats were deeply anesthetized with isoflurane and placed in a stereotaxic apparatus. Stainless steel guide cannulae (Plastics One, Roanoke, VA) were implanted bilaterally into the dorsal hippocampus [coordinates: - 3.2 mm from bregma; ± 2.5 mm lateral; - 1.4 mm deep from dura], according to the atlas of Paxinos and Watson (2005). Rats were monitored and allowed to recover for 7 days after surgery. Prior to training, microinfusion probes (Plastics One) were inserted through the guide cannulae to a point 1 mm below the guide cannulae tips. Compounds were infused bilaterally into the dorsal hippocampus at a rate of 0.25 µl / min for 2 min. Infusion probes were left in place for an additional 1 min to allow solutions to diffuse away from the probe tips.

5.2.4. Inhibitory avoidance training.

All training and testing took place between 12:00 and 16:00 h. The inhibitory avoidance apparatus was a trough-shaped alleyway (91 cm long, 22.9 cm wide at the

top, 7.6 cm wide at the bottom, and 15.2 cm deep) divided into lit (31 cm) and dark (60 cm) compartments by a sliding door that could be lowered through the floor. Each rat was placed in the lit chamber facing the door. When the rat turned completely around, the door was lowered to a height 2 cm above the floor. When the rat again turned toward the door, a timer was started to record the latency to enter the dark chamber (i.e. the training latency). Upon entering the dark chamber, the rat received a brief foot shock (0.2 mA, 0.4 sec) and the door was closed to prevent reentry into the lit chamber. After training or post-training injections, rats were returned to the holding cage. Retention latencies (max of 600 sec) were tested 7 days later using a similar procedure, but without the foot shock. MLA (0.5, 2, or 5 mg/kg) or DHβE (0.5, 2, or 5 mg/kg) were given i.p. 10 min prior to training. Glucose (250 mg/kg) was given i.p. immediately after training.

5.2.5. Foot shock perceptual thresholds.

10 min after i.p. injections of saline or 2 mg/kg MLA or DHβE, rats received a series of foot shocks (each 0.5-sec duration) beginning at 0.1 mA and increasing at 0.05 mA increments with a 30 sec inter-shock interval until a jump response was induced. The lowest shock intensities that caused the rats to flinch and jump were recorded by two independent observers.

5.2.6. Spontaneous alternation testing.

All training took place between 12:00 and 16:00 h. Spontaneous alternation performance was assessed in a 4-arm plus-shaped black Plexiglas maze with an open ceiling. The dimensions of each arm were 45 cm L x 13 cm W x 7 cm H. The

dimensions of the central square-shaped area were 13 cm L x 13 cm W x 7 cm H. The training room contained numerous extramaze visual cues in the form of objects and wall decorations. Rats were placed in a random start arm and were allowed to freely traverse the maze for 20 min. The number and sequence of entries were recorded and alternation performance was calculated. An alternation consisted of visiting all four arms within overlapping sets of five arm visits. Using this procedure, the total number of possible alternations was equal to the number of arm entries minus 4. The percent alternation score is equal to (actual alternations/possible alternations) x 100. Chance performance on this task is 44%. Only data from rats that made at least 10 arm choices (6 possible alternations) were included in the analyses. For experiments with i.p. injections, MLA (0.5, 2, or 5 mg/kg) or DHβE (0.5, 2, or 5 mg/kg) were administered alone or in combination with glucose (250 mg/kg) 30 min prior to testing. For experiments with intrahippocampal injections, MLA (6.75, 13.5, or 27 μg/side) or DHβE (4, 8, or 20 μg/side) were infused alone or in combination with glucose (16.7 nmol/side) beginning 10 min prior to training. Some of the experiments with intrahippocampal injections (i.e. those illustrated in Figure 5.5) utilized a Latin square design in which each rat was tested on four separate occasions with a 3-day rest in between each test. Prior to each test, each rat received a single infusion of a drug, with doses administered in a counterbalanced order.

5.2.7. Perfusion and Brain Slicing.

For analysis of infusion probe placement, brains were collected within two days of the end of behavioral testing. For pCREB immunohistochemistry, brains were collected 30 min after spontaneous alternation testing. Rats were deeply anesthetized

with an overdose injection of sodium pentobarbital (i.p.; Sigma-Aldrich) and then perfused intracardially with 80 ml of 0.1 M phosphate-buffered saline followed by 80 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Rats were decapitated and the brains were removed and placed into 4% paraformaldehyde in 0.1 M PB for ~72 hrs. The brains were transferred to 20% glycerol in 0.1 M PBS for ~48 hrs. For probe placement analysis, frozen sections (40 µM) containing the probe infusion tracts were collected at -30^{o} C with a Leica 1800 cryostat (Leica Microsystems, Wetzlar, Germany). Sections were mounted onto gelatin-coated slides and stained with cresyl violet to visualize probe placements, which were determined using a dissection microscope. For immunohistochemistry, frozen sections (40 µM) just posterior to the injection site were collected at -30^{o} C with a Leica 1800 cryostat. Slices were stored in a cryopreservative solution (250 mM 40 KD polyvinylpyrrolidone, 880 mM sucrose, 30% v/v ethylene glycol, 50 mM sodium phosphate) at -20^{o} C.

5.2.8. Immunohistochemistry.

All steps took place at room temperature and all reactions were performed in duplicate. Slices were washed three times for 10 min each time in 0.05 M PBS initially and between all subsequent steps. Slices were first incubated in blocking solution (1% H_2O_2, 1% normal goat serum, 0.02% triton x-100, 0.05 M PBS) for 10 min. They were transferred to a pre-incubation solution (2% NGS, 0.4% triton x-100, 0.05 M PBS) for 20 min and then incubated overnight in a solution (1% NGS, 0.4% triton x-100, 0.05 M PBS) containing a rabbit primary antibody for Ser-133 phosphorylated CREB (Millipore, Billerica, MA) diluted 1:4000. The next day, the slices were placed for 1 hr in a solution (1% NGS, 0.2% triton x-100, 0.05 M PBS) containing a goat anti-rabbit biotinylated

secondary antibody (Santa Cruz, Santa Cruz, CA). They were next incubated for 30 min with ABC reagent (Vector, Burlingame, CA) in 0.05 M PBS, followed by incubation with DAB substrate (Vector) for 4 min. Slices were mounted onto slides and allowed to dry overnight. The next morning, slices were dehydrated with a graded ethanol series of washes, then coverslipped using DPX mountant (Sigma-Aldrich).

5.2.9. Image Acquisition and Analysis.

Sections were imaged using a Leica DM 6000B/CTR6000 light microscope and a Leica DFC350 FX video camera, which was interfaced to a PC computer. This system was used in conjunction with Image-Pro software (Media Cybernetics, Inc., Bethesda, MD) for image acquisition and for correction of unevenness in illumination across images. Image J software (NIH, Bethesda, MA) was used to quantify the integrated optical density of pCREB staining in area CA1 and the dentate gyrus of the hippocampus. A statistical thresholding method in Image J was used to ensure that only specifically labeled cells were being measured. For each image, the optical density of a nearby region with no or little specific staining was calculated and used for background subtraction.

5.2.10. Statistical Analyses.

All analyses were performed using Statview software. Inhibitory avoidance behavioral results were analyzed using a non-parametric Kruskal-Wallis one-way analysis of variance, followed by Mann-Whitney tests for individual comparisons. The spontaneous alternation behavioral results (except those noted below) and the optical densities of pCREB immunostaining were analyzed using one-way ANOVAs with post

hoc Fisher PLSD tests where appropriate. The spontaneous alternation behavioral results that utilized a Latin square design were analyzed using repeated measures ANOVA with post hoc Fisher PLSD tests where appropriate. Correlations between behavioral results and protein expression were determined by simple linear regression.

5.3. RESULTS

5.3.1. Inhibitory Avoidance Training with Peripheral Injections of Drugs.

Figure 5.1 shows the effects of peripherally-administered nicotinic antagonists on retention of inhibitory avoidance training. Drugs were administered 10 min prior to training. There was a significant main effect of MLA on 7-day retention latencies ($H_{(3,28)}$ = 12.02, $p < .01$). Both the 2 and 5 mg/kg doses of MLA significantly depressed 7-day retention latencies compared to those of saline-injected controls (ps < .05). The 2 mg/kg dose was more effective, significantly depressing 7-day retention latencies compared to the 5 mg/kg dose ($p < .05$). There was also a significant main effect of DHβE on 7-day retention latencies ($H_{(3,28)}$ = 9.60, $p < .05$). The 2 and 5 mg/kg doses of DHβE were similarly effective at depressing 7-day retention latencies compared to saline controls (ps < .05). Neither MLA nor DHβE significantly altered training latencies.

To examine if glucose could attenuate drug-induced impairments in 7-day retention latencies, saline or 2 mg/kg MLA or DHβE were administered 10 min before inhibitory avoidance training in combination with immediate post-training injections of saline or glucose (250 mg/kg). The results are shown in Figure 5.2. Both MLA-saline and DHβE-saline significantly reduced 7-day retention latencies compared to saline-saline controls (ps < .01). Peripheral glucose administration attenuated these drug-induced impairments, but was more effective in reversing DHβE-induced deficits. In the

MLA-glucose group, retention latencies were significantly higher than those in the MLA-saline group, but still significantly reduced compared to those in saline-saline controls (ps < .05). By comparison, retention latencies in the DHβE-glucose group were similar to those in the saline-saline group (p > 0.5), with the group median reaching the 600 sec maximum. Training latencies were not significantly different across groups.

MLA or DHβE-induced impairments in retention latencies may reflect an inability of rats to acquire the learned response. To examine this possibility, two experiments were performed. The first experiment assessed retention latencies using a shorter training-testing interval. Saline or 2 mg/kg MLA or DHβE was injected 10 min prior to training, and retention latencies were tested 1 hr later. All three groups had median 1-hr retention latencies at the maximum of 600 sec, with no significant differences among groups (data not shown). The second experiment examined the effects of the drugs on foot shock perceptual thresholds. Injections of 2 mg/kg MLA or DHβE 10 min prior to foot shock did not significantly alter flinch or jump thresholds compared to those of saline-injected rats. The flinch thresholds were 0.26 ± 0.01 mA (mean ± s.e.m.) for all groups. Jump thresholds ranged from 0.63 ± 0.03 mA in the saline and DHβE groups to 0.65 ± 0.03 in the MLA group.

5.3.2. Spontaneous alternation testing.

5.3.2.1. Peripheral injections of drugs.

Figure 5.3 shows the effects of peripherally-administered nicotinic antagonists on spontaneous alternation scores. MLA or DHβE given 30 min before testing impaired alternation scores, with effective doses similar to those observed in the inhibitory avoidance task ($F_{(3,28)} = 3.09$, $p < .05$ for MLA; $F_{(3,28)} = 3.34$, $p < .05$ for DHβE). For the

MLA experiment, the intermediate dose of 2 mg/kg, but not lower or higher doses, significantly reduced alternation scores compared to saline-injected controls ($p < .05$). In contrast, MLA had no significant effect on the number of arm entries during testing, which ranged from 24.6 ± 2.8 (mean ± s.e.m.) in the saline group to 26.1 ± 2.6 in the 2 mg/kg MLA group. For the DHβE experiment, doses of 2 and 5 mg/kg were about equally effective at impairing alternation scores compared to saline controls (ps < .05). The number of arm entries decreased with increasing doses of DHβE, ranging from mean values of 23.3 ± 1.3 in the saline group to 18.5 ± 1.9 in the 5 mg/kg DHβE group. However, this was not a statistically significant effect.

To determine if glucose could attenuate drug-induced impairments in spontaneous alternation scores, MLA or DHβE (2 mg/kg) was administered alone or in combination with glucose (250 mg/kg) 30 min before testing (Figure 5.4). MLA and DHβE significantly impaired alternation scores compared to saline-injected controls (ps < .01). Glucose had no significant effect on attenuating MLA-induced deficits; rats treated with MLA-glucose had alternation scores similar to those in the MLA group ($p > 0.5$) and significantly lower than those of saline controls ($p < .05$). However, glucose reversed DHβE-induced impairments in alternation performance, raising scores well above those of rats administered DHβE alone ($p < .05$). The number of arm entries were significantly reduced in the DHβE (17.5 ± 1.5) and DHβE-glucose (17.1 ± 0.8) groups compared to saline controls (23.4 ± 1.4; ps < .01). Therefore, the effect of glucose was restricted to DHβE effects on alternation scores and did not extend to drug effects on locomotor activity. The number of arm entries in the other groups were not significantly different from those in the saline group.

5.3.2.2. Intrahippocampal injections of drugs.

Figure 5.5 shows the effects of intrahippocampally-administered nicotinic antagonists on spontaneous alternation scores. To reduce the number of rats subjected to surgical procedures, a Latin square design was used here but not in subsequent experiments with glucose. Drugs were infused beginning 10 min before testing. Both MLA and DHβE infusions significantly impaired alternation scores ($F_{(3,24)} = 3.02$, $p < .05$ for MLA; $F_{(3,24)} = 3.17$, $p < .05$ for DHβE). MLA doses of 6.75 and 13.5 µg/side were equally effective at impairing alternation scores compared to those of aCSF-injected controls (ps < .05). However, MLA lost its effectiveness in impairing alternation scores at the highest dose of 27 µg/side. DHβE was ineffective at the lowest dose of 4 µg/side, but significantly impaired alternation performance at higher doses of 8 and 20 µg/side (ps < .05 vs. aCSF). Neither MLA nor DHβE infusions significantly altered the number of arm entries. The mean numbers of arm entries were slightly lower than those seen with peripheral injections, ranging from 19.4 ± 2.8 to 23.1 ± 2.7 in the MLA experiment and 19.1 ± 2.4 to 21.2 ± 1.4 in the DHβE experiment.

The effects of intrahippocampal glucose on attenuating drug-induced deficits largely paralleled the results seen with peripheral injections. Glucose (16.7 nmol/side) was combined with the lowest effective doses of the nicotinic antagonists and infused 10 min before alternation testing. As shown in Figure 5.6, glucose had no effect on attenuating MLA-induced impairments in alternation performance. Both the MLA and MLA-glucose groups had significantly depressed alternation scores (ps < .05 vs. aCSF), which were not significantly different from each other ($p > 0.5$). In contrast, glucose reversed DHβE-induced impairments in alternation scores. Although DHβE alone

produced significant deficits in alternation scores ($p < .05$ vs. aCSF), the DHβE-glucose group had similar scores compared to aCSF ($p > 0.5$). Unlike the pattern of results seen with peripheral injections of drugs, there was a trend for enhancement in alternation scores following infusion of intrahippocampal glucose alone ($p < .06$ vs. aCSF). The number of arm entries were similar across groups, ranging from 19.1 ± 3.0 in the DHβE-glucose grouop to 24.6 ± 3.8 in the MLA-glucose group.

5.3.3. pCREB Staining

Immunostaining levels of pCREB were examined 30 min after spontaneous alternation testing in rats receiving intrahippocampal infusions of drugs (corresponding to Figure 5.6). As shown in Figure 5.7, pCREB staining levels in area CA1 paralleled the behavioral results. Both MLA and DHβE impaired pCREB staining compared to aCSF-injected controls (ps < .05). Glucose reversed the DHβE- but not MLA-induced pCREB deficits ($p > 0.5$ DHβE vs. aCSF; $p < .05$ MLA vs. aCSF). Unlike with the behavioral results, there was no trend for enhancement of pCREB staining by intrahippocampal glucose alone ($p > 0.5$). In contrast to the results for area CA1, there were no significant effects of drug treatments on pCREB staining levels in the dentate gyrus (data not shown).

As shown in Figure 5.8, there was a significant positive correlation between area CA1 pCREB staining levels and spontaneous alternation scores in rats receiving MLA-glucose injections ($r = 0.90$, $p < .01$), and a trend for significance in rats receiving MLA alone ($r = 0.69$, $p = .09$). There were no correlations or trends in any of the other groups (ps > 0.2).

5.4. DISCUSSION

5.4.1. Effects of nicotinic antagonists on memory.

Peripheral injections of MLA or DHβE impaired memory when tested 7 days after inhibitory avoidance training, supporting the view that inhibition of cholinergic signaling through either α7 or α4β2 nicotinic receptors inhibits the formation of long-lasting memories. Previous studies in rats and mice have shown that pre-training peripheral administration of the centrally-acting nicotinic antagonist mecamylamine impairs later retention for inhibitory avoidance training, whereas the peripherally-acting nicotinic antagonist hexamethonium has no effect (Chiappetta and Jarvik, 1969; Glick and Greenstein, 1972; Goldberg et al., 1971; Ragozzino and Gold, 1991; Rush and Streit, 1992). The present results extend these findings, suggesting that mecamylamine could exert its behavioral effects through inhibition of α4β2 and/or α7 receptors in the brain.

In the present study, the administration of the drugs prior to instead of after inhibitory avoidance training leaves open the possibility that the drugs produced apparent amnesia by altering factors other than memory, such as attention, motivation, or sensorimotor processes. However, a few pieces of evidence indicate that behavioral differences due to effects on non-mnemonic variables are unlikely. First, neither MLA nor DHβE altered training latencies or thresholds for foot shock sensitivities, suggesting that these measures of sensorimotor processes were intact during training. Second, the high memory scores in MLA- or DHβE-injected rats tested 1 hr after training indicate the drugs did not interfere with the ability of the rats to learn and initially remember the training experience. However, differences between saline- and drug-injected groups

could have been masked by a ceiling effect, given that the median 1-hr retention latencies in all groups reached the maximum of 600 sec.

Peripheral administration of MLA or DHβE also impaired performance in the spontaneous alternation task. These results were remarkably similar to the effects of the antagonists on retention performance in the inhibitory avoidance task, and were consistent with past findings showing that peripheral injections of mecamylamine, but not the peripherally acting hexamethonium, impair alternation performance (Ragozzino and Gold, 1991). In both the spontaneous alternation and inhibitory avoidance tasks, MLA-induced impairments followed a U-shaped pattern, in which the middle dose of 2 mg/kg was most effective at impairing behavioral performance. In contrast, both 2 and 5 mg/kg doses of DHβE were similarly effective at impairing performance in these behavioral tasks. The U-shaped pattern observed for MLA-induced deficits may relate to non-specific effects of the drug at higher doses.

Direct infusions of MLA or DHβE into the dorsal hippocampus also impaired performance in the spontaneous alternation task, suggesting that the drugs work directly in the brain to impair spatial working memory processes. These results are consistent with prior behavioral studies of spatial working memory in the appetitive radial arm maze task, where infusions of MLA or DHβE directly into the hippocampus, amygdala, or frontal cortex, significantly increased working memory errors (Addy et al., 2003; Bettany and Levin, 2001; Chan et al., 2007; Levin et al., 2002; Nott and Levin, 2006). Interestingly, infusions of DHβE into the mediodorsal thalamic nucleus actually reduced working memory errors during radial arm maze testing (Cannady et al., 2009). Opposing effects in different brain regions of MLA or DHβE have also been observed in

trace fear conditioning (Raybuck and Gould, 2010), suggesting some regional variability in the actions of these drugs on learning and memory. In the present study, the effects of peripheral administration of MLA or DHβE on alternation scores were similar to those following intrahippocampal infusions, consistent with the possibility that the effects on memory of the peripherally-injected drugs may be mediated by hippocampal actions.

5.4.2. Ability of glucose to reverse drug-induced memory impairments.

In the inhibitory avoidance task, post-training injections of glucose were more effective at reversing DHβE- than at reversing MLA-induced memory deficits. Likewise, in the spontaneous alternation task, co-administration of glucose with the antagonists reversed DHβE- but not MLA-induced memory impairments, regardless of whether the drug/glucose combinations were administered peripherally or directly into the hippocampus. Previous work has shown that glucose reverses the memory-impairing effects of mecamylamine in both the inhibitory avoidance and spontaneous alternation tasks. However, glucose does not attenuate mecamylamine-induced decreases in locomotor activity, such as reductions in the number of arm entries during spontaneous alternation testing (Ragozzino and Gold, 1991; Ragozzino et al., 1994). In the present study, the results obtained with peripheral DHβE administration parallel those seen previously with mecamylamine. DHβE produced memory impairments and decreased the number of arm visits during spontaneous alternation testing; glucose attenuated the memory but not motor deficits. In contrast, MLA-induced memory impairments were not attenuated by glucose and followed a U-shaped dose-response function not seen previously with mecamylamine. MLA also had no significant effect on spontaneous alternation arm entries. The similarities in the behavioral effects of mecamylamine and

DHβE but not MLA may be a consequence of the functional properties of mecamylamine. Although mecamylamine is a nonselective nicotinic antagonist, it is a more potent and long-lasting inhibitor of β subunit-containing receptors (Papke et al., 2001). Therefore, the behavioral effects of mecamylamine are more likely mediated by inhibition of α4β2 and/or other β subunit-containing receptors, as opposed to α7 receptors.

One possible explanation for why glucose reverses DHβE- but not MLA-induced behavioral deficits is that the memory-improving actions of glucose may rely more on α7- as opposed to α4β2-mediated signaling processes. For example, glucose may selectively up-regulate acetylcholine release in regions with high densities of α7 receptors. Previous studies suggest that glucose is targeted to particular neural systems in response to task demands, where it may enhance local acetylcholine release (cf. Gold, 2004). There are high concentrations of α7 receptors in brain areas in which direct glucose injections lead to memory improvement. For example, the hippocampus has particularly dense levels of α7 receptors, which have a distinct regional localization and cell type distribution compared to other hippocampal acetylcholine receptors, including α4β2 receptors (Fabian-Fine et al., 2001; Gotti and Clementi, 2004; Tribollet et al., 2004). By contrast, Pych et al. (2006) showed that injections of glucose into the dorsal striatum, which has relatively low levels of α7 receptors but an abundance of α4β2 receptors (Gotti and Clementi, 2004; Tribollet et al., 2004), failed to enhance learning of a striatum-sensitive maze in which learning is based on egocentric responses.

Glucose may also selectively modulate α7-mediated signaling processes due to the unique functional properties of these receptors. α7 receptors have the highest

calcium permeability of any known nicotinic receptor subtype (Castro and Albuquerque, 1995; Séguéla et al., 1993; Shen and Yakel, 2009). Calcium influx through these receptors plays an important role in the presynaptic release of GABA, glutamate, and other neurotransmitters in the brain (Shen and Yakel, 2009; Sher et al., 2004; Wonnacott et al., 2006). Glucose may potentiate these responses through actions mediated by presynaptic ATP-dependent potassium (K-ATP) channels. A number of studies indicate that regulation of K-ATP channel activity is important to the memory-enhancing effects of glucose (Stefani and Gold, 1998, 2001; Stefani et al., 1999; Rashidy-Pour, 2001). Another unique property of α7 receptors is that they have a high affinity for choline, which functions as a full and selective agonist of these receptors (Alkondon et al., 1997; Mike et al., 2000; Uteshev et al., 2003). Glucose may modulate the activity of α7 receptors by regulating local extracellular choline levels. Previous studies indicate that glucose regulates high-affinity choline uptake and can act synergistically to improve memory when administered together with choline (Kopf et al., 2001; Micheau et al., 1995; Messier et al., 1990). However, the effects of glucose on extracellular choline levels, in the context of memory enhancement, have not been directly investigated.

5.4.3. Effects of nicotinic antagonists and glucose on CREB activation.

Intrahippocampal injections of MLA or DHβE reduced pCREB levels in area CA1 but not the dentate gyrus, perhaps because of insufficient diffusion of the drugs to this hippocampal region. Co-administration of glucose reversed DHβE- but not MLA-induced pCREB deficits in area CA1, paralleling the behavioral results. The ability of glucose to reverse DHβE-induced deficits in pCREB activation supports prior work showing that

peripheral glucose or intrahippocampal lactate administration modulates hippocampal CREB phosphorylation in the context of memory enhancement (Morris and Gold, 2012b; Suzuki et al., 2011). Glucose infusions into area CA1 have also been shown to activate components of the mTOR pathway while improving memory (Dash et al., 2006).

Glucose did not attenuate MLA-induced deficits in pCREB activation in area CA1, and levels of pCREB in MLA-injected rats were correlated with individual differences in alternation performance. These results support the hypothesis that α7 signaling pathways involving CREB activation may be important to the memory-enhancing effects of glucose. Previous studies have shown that chronic changes in CREB levels were correlated with spatial working memory performance in the radial arm maze (Rendeiro et al., 2012; Williams et al., 2008). However, based on the time of drug administration in the present study, it is unlikely that the acute changes in CREB phosphorylation could initiate transcriptional events quickly enough to alter behavioral performance. More likely, CREB activation here serves as a good marker of upstream molecular processes that may play a more direct role in modulating spatial working memory processes.

5.4.4. Conclusions.

The present results indicate that glucose reverses deficits in memory and CREB phosphorylation produced by the α4β2 receptor antagonist DHβE but not the α7 receptor antagonist MLA. The ineffectiveness of glucose at reversing MLA-induced deficits in both behavioral and molecular measures suggests that the memory-enhancing effects of glucose involve its ability to activate downstream α7 receptor signaling. Although α4β2 receptors appear to be less important in this regard, the

current results do not rule out the possibility that other acetylcholine receptor subtypes (e.g. muscarinic receptors) or neurotransmitter systems may also play a role. Given the central function of glucose in brain metabolism, it seems likely that glucose modulates cognition by multiple mechanisms, perhaps dependent on the interaction of brain area, memory task, and other variables.

5.5. FIGURES

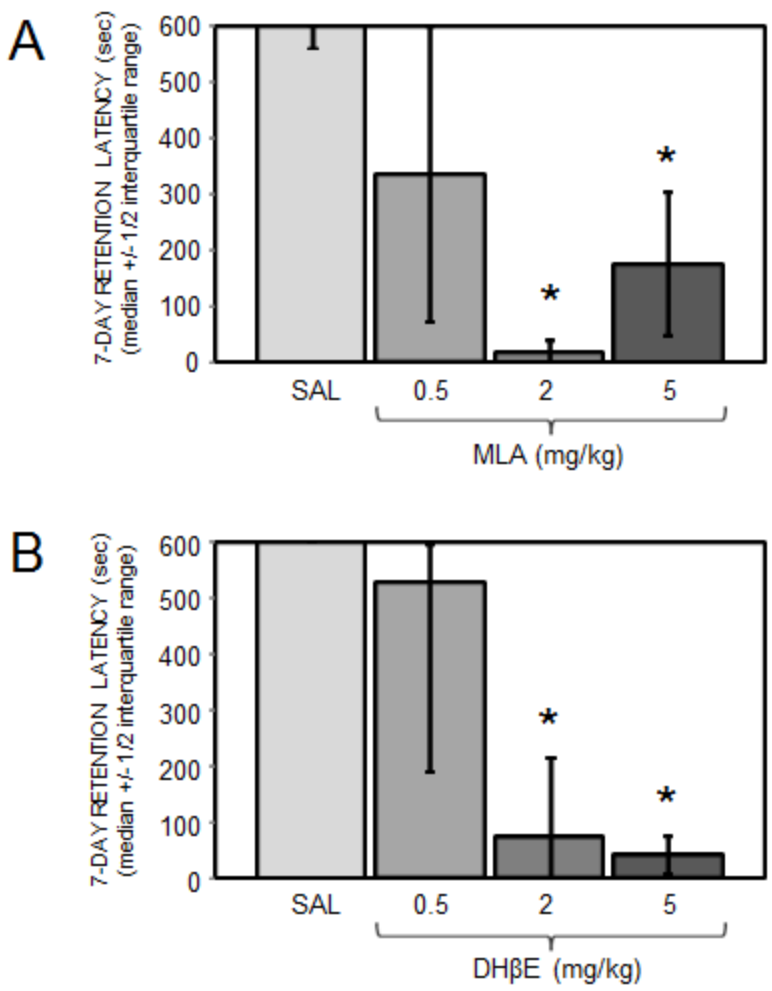

Figure 5.1. Effects of peripherally-administered nicotinic antagonists on inhibitory avoidance memory tested 7 days after training.

(A) MLA doses of 2 and 5, but not 0.5 mg/kg, significantly impaired memory. (*) ps < .05 vs. saline. Ns = 8. (B) DHβE doses of 2 and 5, but not 0.5 mg/kg, significantly impaired memory. (*) ps < .05 vs. saline. Ns = 8.

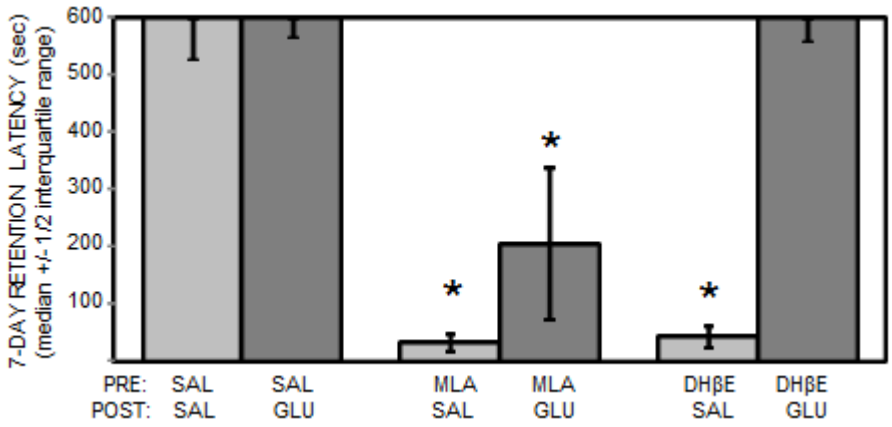

Figure 5.2. Glucose attenuation of drug-induced memory impairments after inhibitory avoidance training.

Pre-training administration of MLA or DHβE (2 mg/kg), together with post-training saline injections, produced deficits in memory tested at 7 days after training. Post-training glucose attenuated the drug-induced memory impairments, but was more effective at reversing the impairments induced by DHβE. (*) ps < .05 vs. saline-saline. Ns = 8.

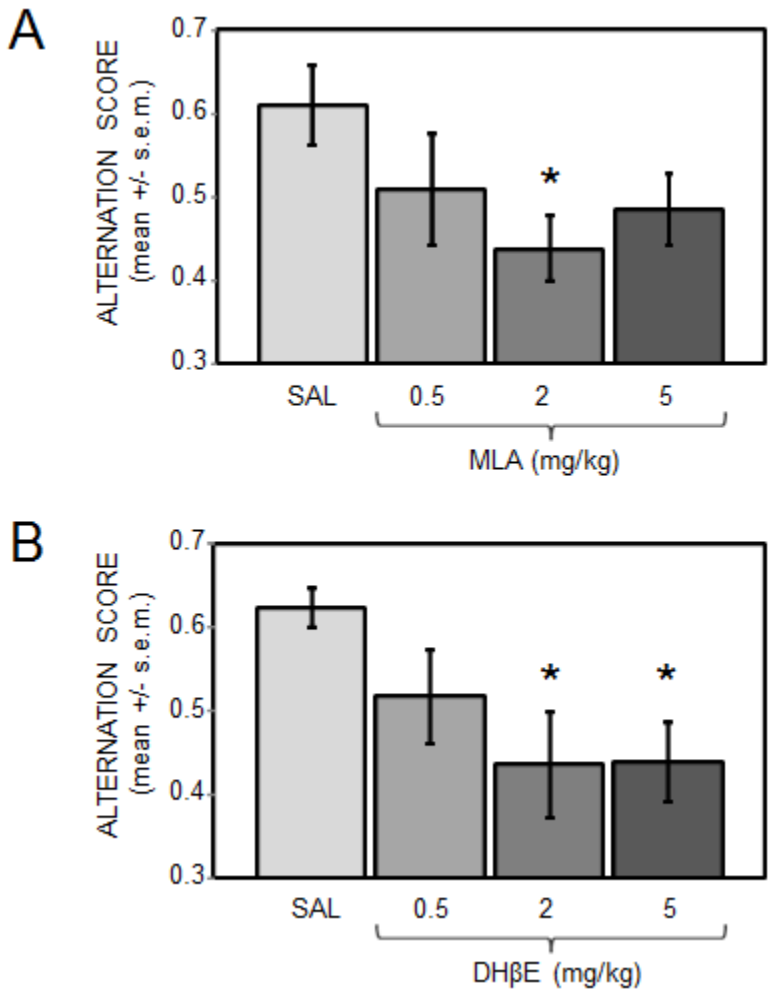

Figure 5.3. Effects of peripherally-administered nicotinic antagonists on spontaneous alternation spatial working memory scores.

(A) An MLA dose of 2, but not 0.5 or 5 mg/kg, significantly impaired alternation scores. (*) $p < .05$ vs. saline. Ns = 8. (B) DHβE doses of 2 and 5, but not 0.5 mg/kg, significantly impaired alternation scores. (*) $ps < .05$ vs. saline. Ns = 8.

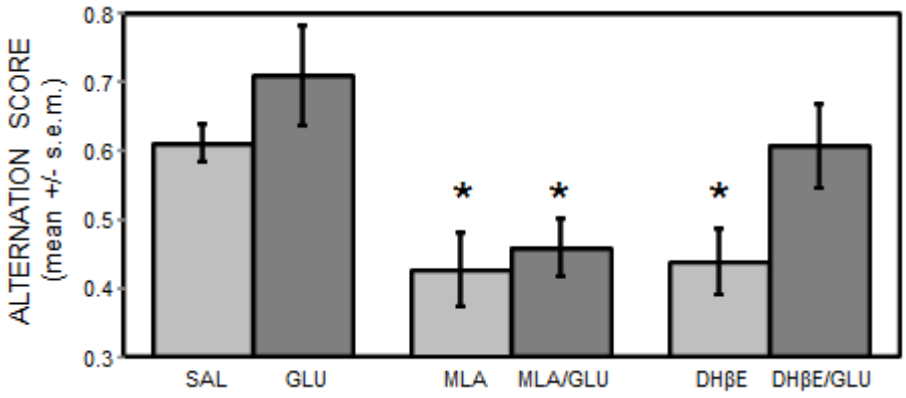

Figure 5.4. Glucose attenuation of drug-induced working memory impairments in the spontaneous alternation task.

MLA or DHβE (2 mg/kg) produced deficits in alternation scores. Co-administration of glucose with the antagonists attenuated DHβE but not MLA-induced deficits. (*) ps < .05 vs. saline. Ns = 8.

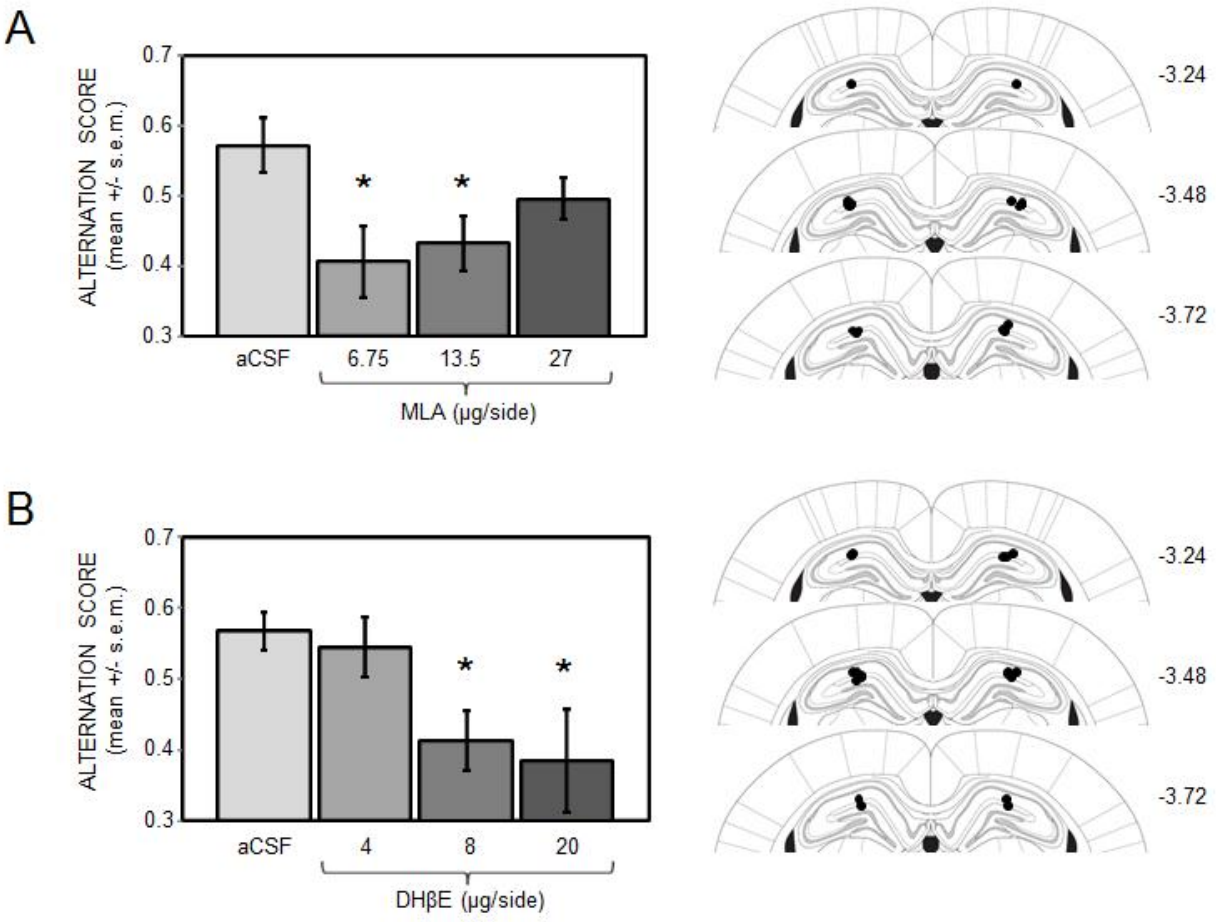

Figure 5.5. Effects of intrahippocampally-administered nicotinic antagonists on working memory assessed in a spontaneous alternation task.

(A) MLA doses of 6.75 and 13.5, but not 27 µg/side, significantly impaired alternation scores. (*) ps < .05 vs. aCSF. N = 9. (B) DHβE doses of 8 and 20, but not 4 µg/side, significantly impaired alternation scores. (*) ps < .05 vs. aCSF. N = 9. To the right of each graph is an illustration of the infusion sites targeting the dorsal hippocampus for each experiment. Filled circles represent tips of infusion tracts. Numbers refer to distance in mm posterior to bregma. Adapted with permission from Paxinos and Watson (2005).

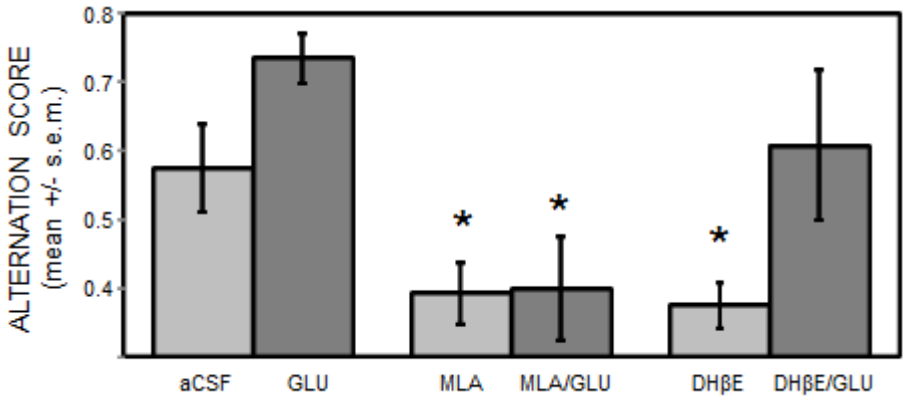

Figure 5.6. Glucose attenuation of impairments in spontaneous alternation memory scores produced by intrahippocampally-administered antagonists.

MLA (6.75 µg/side) or DHβE (8 µg/side) produced deficits in alternation scores. Co-administration of glucose with the antagonists attenuated DHβE but not MLA-induced deficits. There was a trend for enhancement in alternation scores following administration of glucose alone. (*) ps < .05 vs. aCSF. Ns = 8 for aCSF and GLU. Ns = 7 for all other groups.

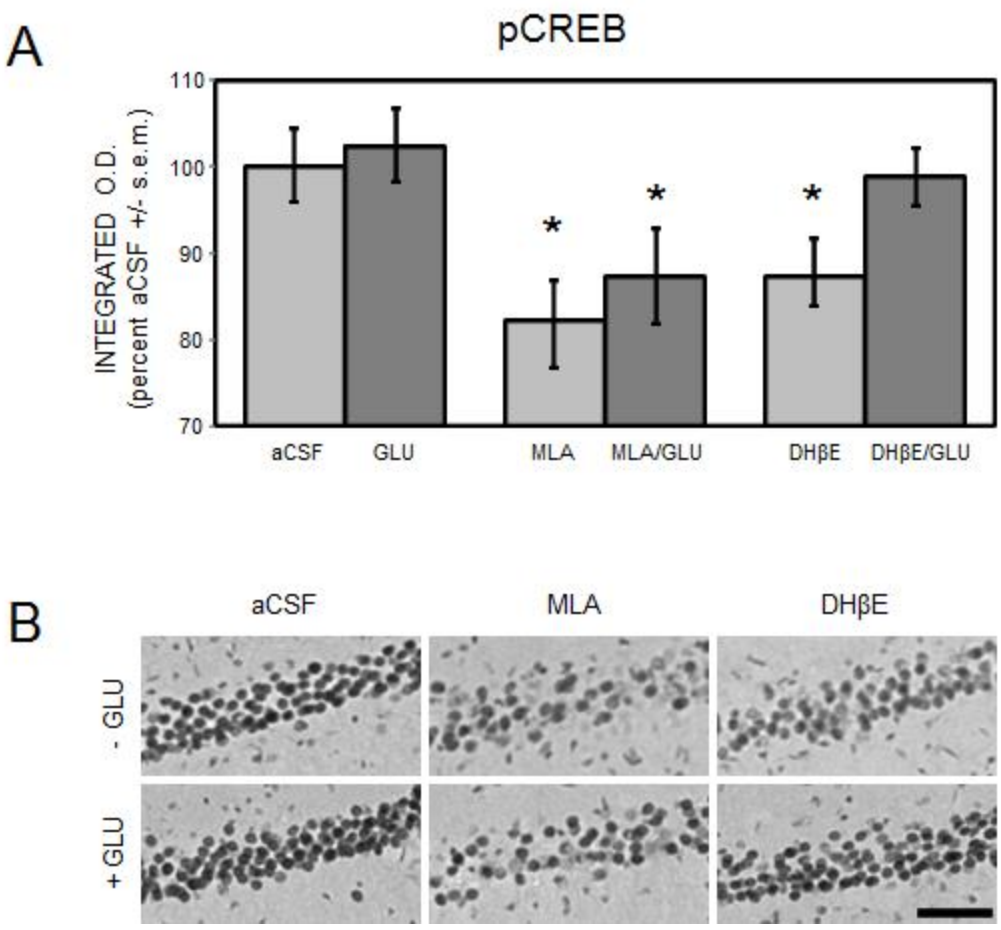

Figure 5.7. Differences in pCREB immunoreactivity in area CA1 following spontaneous alternation testing with intrahippocampal administration of drugs.

(A) MLA (6.75 µg/side) or DHβE (8 µg/side) reduced pCREB levels. Co-administration of glucose with the antagonists attenuated DHβE but not MLA-induced pCREB deficits. (*) ps < .05 vs. aCSF. Ns = 8 for aCSF and GLU. Ns = 7 for all other groups. (B) Representative photomicrographs of pCREB immunostaining in area CA1. Scale bar = 100 microns.

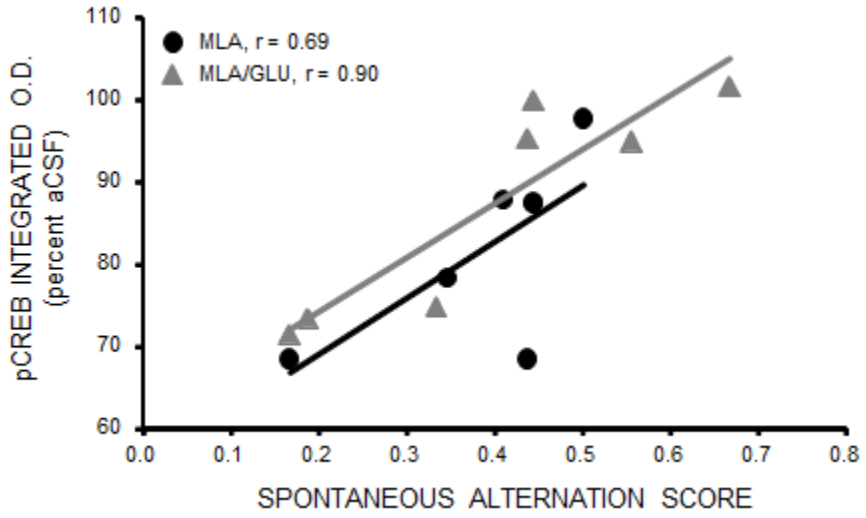

Figure 5.8. Correlations between area CA1 pCREB immunostaining and spontaneous alternation scores.

There was a significant correlation in rats receiving intrahippocampal injections of MLA-glucose ($p < .01$), and a trend for a correlation in rats receiving MLA alone ($p = .09$).

CHAPTER 6: CONCLUSIONS

The main objective of this dissertation was to examine how age-related deficits in blood glucose responses to epinephrine could alter central neurobiological processes and produce memory impairments. The major hypothesis was that an uncoupling between peripheral epinephrine and glucose release in old rats causes memory impairments by altering downstream cholinergic signaling processes in the brain. This hypothesis, as well as the accompanying model presented in Figures 1.1 and 1.2, was supported by a variety of data.

Compared to young adult rats, old rats exhibited rapid forgetting in the inhibitory avoidance task, which correlated with neurochemical and molecular deficits (Chapters 2, 3, and 4). *In vivo* microdialysis measures indicated that old rats had significantly reduced hippocampal acetylcholine release at baseline and in response to training. Immunohistochemistry measures 30 min after training showed that old rats had significantly reduced pCREB levels in the hippocampus and amygdala. The extensive age-related depletion in acetylcholine release likely made a large contribution to pCREB deficits, even though CREB phosphorylation is downstream of a variety of neurotransmitters and growth factors. Post-training glucose injections significantly reversed age-related memory impairments while attenuating deficits in hippocampal acetylcholine release and CREB phosphorylation. In contrast, post-training epinephrine injections were less effective in each of these cases. The greater effectiveness of glucose was most likely because it could bypass the age-related uncoupling between peripheral epinephrine and glucose release. The only measure at which epinephrine was more effective than glucose was at attenuating age-related deficits in CREB

phosphorylation in the amygdala. This is likely because epinephrine can stimulate other pathways to CREB phosphorylation independent of its effects on blood glucose levels. Together, these results suggest that age-related changes in peripheral epinephrine and glucose release can alter neural functions, depressing local acetylcholine release and changing regional patterns of CREB phosphorylation, perhaps leading to memory impairments.

Unlike in the other chapters, the experiments in Chapter 5 utilized only young adult rats, lacking direct comparisons with old rats. Instead, these experiments effectively modeled age-related memory impairments in young rats by injecting them with one of two nicotinic acetylcholine receptor antagonists: the α7 receptor antagonist MLA or the α4β2 receptor antagonist DHβE. Rats receiving either MLA or DHβE had impairments in behavioral and molecular measures similar to those seen in old rats. In the inhibitory avoidance task, rats receiving either antagonist had a strong memory of the training experience when tested 1-hr but not 7 days later, mimicking age-related forgetting. In the spontaneous alternation task, rats receiving either antagonist had alternation scores near chance performance, similar to the deficits observed in aged rats. In addition, MLA and DHβE produced deficits in hippocampal CREB phosphorylation following spontaneous alternation testing. Interestingly, glucose injections were much more effective at attenuating the memory and CREB deficits induced by DHβE as opposed to those induced by MLA. These results suggest that memory enhancement and reversal of age-related memory impairments by glucose involve its ability to activate downstream α7 receptor signaling. This hypothesis is further supported by data not shown in this dissertation, in which MLA blocked the

memory-enhancing effects of glucose in the inhibitory avoidance and spontaneous alternation tasks. However, corresponding experiments with DHβE were not completed.

Taken together, the studies presented in this dissertation provide a potential mechanism for age-related increases in the rate of forgetting (see Figure 1.2). According to this model, age-related deficits in blood glucose rises in response to endogenous epinephrine release reduce the supply of glucose to particular neural systems important for memory. This diminished neuroendocrine response limits cholinergic signaling processes, including reducing activation of α7 nicotinic acetylcholine receptors. Depressed nicotinic signaling impairs a variety of downstream molecular processes, including CREB phosphorylation. Any or all of the deficits along this pathway could produce age-related memory impairments.

An open question is why blood glucose levels lose their sensitivity to epinephrine in old rats. One clue is that old rats actually have higher circulating epinephrine in response to training or stress, perhaps representing an impaired physiological mechanism in the liver for producing increases in blood glucose levels. This hypothesis is supported by evidence that old rats have reduced liver glycogen levels and impaired mobilization of glycogen following epinephrine injections compared to young rats. However, blood glucose levels are regulated by a number of other hormones, including insulin, glucagon, and corticosterone. Evaluating age-related changes in these hormones, as well as their interactions in regulating glucose homeostasis, may provide greater insight into deficits in blood glucose rises in response to stress or training.

Although both epinephrine and glucose support improved memory in old rats, glucose appears to be more effective. This is not surprising given that glucose is the

primary energy source for the brain under normal physiological conditions. Therefore, one strategy for attenuating or reversing age-related cognitive impairments in humans may be to administer oral glucose supplements. A number of studies have shown that glucose but not the artificial sweetener saccharin improves cognitive functions in elderly individuals, particularly on tasks where there are age-related deficits. However, there are a variety of potential problems associated with glucose supplementation in elderly people. First, glucose only works when it is administered close to the time of cognitive testing. In everyday life, it would be difficult to predict the times associated with high cognitive demand in which glucose would be most beneficial. Second, a relatively large percentage of older individuals have impaired blood glucose regulation, which can be comorbid with chronic diseases that produce memory impairments. Administering glucose to these individuals could exacerbate chronic conditions and be potentially dangerous. Finally, glucose or other carbohydrates eventually broken down into glucose are an important part of our diets. Coordinating glucose supplementation with normal dietary intake would be complicated, requiring highly compliant patients.

Targeting cholinergic signaling, and in particular nicotinic α7 receptors, may be another strategy for attenuating or reversing age-related cognitive impairments. However, drugs that enhance acetylcholine levels in the brain or that activate specific muscarinic or nicotinic acetylcholine receptor subtypes have only been mildly successful at treating age-related memory pathologies. Acetylcholinesterase inhibitors remain the only class of cholinergic drugs that have significantly improved cognitive, behavioral, and other outcome measures in large clinical trials of patients with Alzheimer's disease. Even so, cholinesterase inhibitors are only expected to "delay worsening of symptoms

for 6 to 12 months, on average, for about half the people who take them," according to the Alzheimer's Association. Cholinesterase inhibitors also provide little benefit to patients with mild cognitive impairment, which often precedes the development of Alzheimer's disease. The large majority of drugs in development for the treatment of Alzheimer's disease and other age-related memory pathologies target the associated inflammation, insulin resistance, or abnormal accumulation of plaques or tangles, rather than directly modulating cholinergic signaling processes.

Cholinergic drugs are just one example of memory-enhancing agents that have shown little effectiveness in treating the symptoms or delaying the progression of human memory pathologies. Other such agents include phosphodiesterase inhibitors and drugs that target different neurotransmitter systems. The ineffectiveness of these pharmacological treatments may relate to the timing of drug administration. These drugs are usually administered routinely to achieve more or less constant levels in the brain. Similar to the problems associated with chronic glucose administration discussed above, this regimen ignores important concepts related to memory modulation, i.e. memory enhancing agents work best when administered at or near the time of a particular experience. Chronically increasing levels of these drugs may provide some benefit, especially in pathologies where there are significant impairments in basal functions. However, this could also result in a diminished sensitivity to modulatory processes, possibly reducing the durability of newly formed memories.

The current trend is to develop memory-enhancing drugs that have more and more specific mechanisms of action. For example, a variety of nicotinic α7 receptor agonists are currently being developed and tested for the potential treatment of the

symptoms of Alzheimer's disease and related cognitive deficits. This approach may lead to fewer side effects and higher therapeutic indices, but is unlikely to produce significant memory benefits due to its narrow focus. Memory formation is modulated by multiple interconnected or independent pathways and is dependent on the interaction of brain area, memory task, and other variables. Agents such as α7 agonists that target specific receptor subtypes may show a high degree of efficacy in rodent models because these are controlled environments in which relatively subtle changes in memory function can be measured. However, these benefits are unlikely to translate into clinical settings.

The development of more broad-based treatment strategies may be necessary to mitigate age-related memory impairments in humans. Instead of focusing on drugs that target very specific pathways, more attention should be given to global modulators of memory function, such as peripheral hormones and neuroendocrine systems. The results presented in this dissertation suggest that an important component of age-related memory impairments may be reduced blood glucose responses to epinephrine. Therefore, improving the sensitivity of this neuroendocrine response may represent a viable strategy for attenuating age-related impairments in memory.

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